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THE EFFECT OF MCT1 AND MCT4 SILENCING ON PROSTATE CANCER SURVIVAL AND AGGRESSIVENESS

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Dissertação de Candidatura ao grau de Mestre em Oncologia (Especialização Oncologia Molecular) submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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“Pedras no caminho? Guardo-as todas, um dia vou construir um castelo…”

Fernando Pessoa

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ABSTRACT / RESUMO

Abstract

The preference for a glycolytic metabolism as mean of energy production, even in the presence of oxygen ("Warburg effect"), is a widely described phenomenon in tumors. The higher glycolytic rates verified in tumour cells leads to an increase in lactate production, which transport out of the cell, performed by monocarboxylate transporters (MCTs) with co-transport of a proton, contributes to an increase in cell proliferation, invasion and survival, by causing the acidification of tumor microenvironment. Studies have been exploring the role of MCTs in the metabolism of tumor cells, however the role of MCTs is controversial and poorly explored prostate cancer.

The aim of this work was to understand the role of MCTs on prostate cancer cells survival and on cancer cell characteristic features of aggressiveness. To achieve our goals, we performed a detailed characterization of MCTs expression and other metabolic markers in two prostate cancer cell lines, with distinct phenotypes of aggressiveness. Furthermore, we studied the effects of MCT silencing on prostate cell models in what regards to cell survival, proliferation, migration and cellular energetic metabolism. Our study showed that, not only MCTs are expressed on prostate tumor cells, but also its silencing affects cell growth and metabolism, as well as proliferation and migration capacity in *in vitro* models. Importantly, there was an effective reduction of tumor growth *in vivo*, when MCTs were silenced.

Although further studies are needed, our results demonstrate the potential of MCTs as therapeutic targets in prostate cancer.

Resumo

A preferência por um metabolismo glicolítico pelas células tumorais como meio para produção de energia, mesmo na presença de condições normais de oxigénio ("efeito de Warburg"), é um fenómeno vastamente descrito em tumores. As altas taxas de glicólise verificadas nas células tumorais levam a um aumento na produção de lactato, cujo transporte para o exterior da célula é efetuado pelos transportadores de monocarboxilatos (MCTs) com co-transporte de um protão, contribuindo para um aumento na proliferação, invasão e sobrevivência das células, causando a acidificação do microambiente tumoral. Estudos têm investigado o papel dos MCTs no metabolismo das células tumorais, contudo esse papel é ainda controverso e pouco explorado no cancro da próstata.

O objetivo deste trabalho foi entender o papel dos MCTs na sobrevivência e características de agressividade das células tumorais de próstata. Para atingir os nossos objetivos, realizámos uma caracterização detalhada da expressão dos MCTs e outros marcadores metabólicos em duas linhas celulares de cancro de próstata, com diferentes fenótipos de agressividade. Além disso, estudámos o efeito do silenciamento dos MCTs em modelos celulares de próstata no que respeita à sobrevivência, proliferação, migração e metabolismo energético celulares. O nosso estudo demonstrou que, não só os MCTs estão expressos nas células tumorais de próstata, como também que o seu silenciamento afeta a viabilidade e o metabolismo celulares, assim como a capacidade de proliferação e migração em modelos *in vitro*. Mais importante, houve uma redução efetiva no crescimento do tumor *in vivo*, quando os MCTs foram silenciados.

Apesar de serem necessários estudos mais aprofundados, os nossos resultados demonstraram o potencial dos MCTs como alvos terapêuticos no cancro da próstata.

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ABBREVIATIONS LIST

Abbreviations List

PSA – Prostate Specific Antigen

DRE – Digital Rectal Exam

FDA – Food and Drug Administration

TNM – Tumor-Node-Metastasis

AJCC – American Joint Committee on Cancer

UICC – International Union for Cancer Control

RP – Radical Prostectomy

TCA – Tricarboxylic Acid

ATP - Adenosine Tri-Phosphate

NAD – Nicotinamide Adenine Dinucleotide

FdG-PET – ^{18}F-fluorodeoxyglucose Positron Emission Tomography

FDG – 2- (18F) -Fluoro-2-Deoxy-D-Glucose

ROS – Reactive Oxygen Species

HIF – Hypoxia Inducible Factor

HREs – Hypoxic-Response Elements

VEGF – Vascular Endothelial Growth Factor

PDK – Pyruvate Dehydrogenase Kinase

PDH – Pyruvate Dehydrogenase

GLUT – Glucose Transporter

HK – Hexokinase

PK – Pyruvate Kinase

LDH – Lactate Dehydrogenase

CA – Carbonic Anhydrase

MCT – Monocarboxylate Transporter

NHE – Na^+/H^+ Exchanger

AE – Anion Exchanger

TGF – Transforming Growth Factor

SLC – Solute Carrier

TMD – Transmembrane Domain

UTR – Untranslated Region

RPE – Retinal Pigment Epithelium

NCBI – National Center for Biotechnology Information

EMMPRIN – Extracellular Matrix Metalloproteinase Inducer

AMACR – Alpha-Methylacyl-CoA Racemase

CHC – α-cyano-4-hydroxycinnamate

siRNA – small-interference RNA

ATCC – American Type Culture Collection

RPMI – Roswell Park Memorial Institute

FBS – Fetal Bovine Serum

ICC – Immunocytochemistry

DAB – Diamonobenzidine

PBS – Phosphate-Buffer Saline

SRB – Sulforhodamine B

BrdU – 5-bromo-2´-deoxyuridine

GOD – Glucose Oxidase

AP – Aminophenazone

POD – Peroxidase

ICVS – Instituto de Investigação em Ciências da Vida e da Saúde

CAM – Chorioallantoic Membrane

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CHAPTER 1: GENERAL INTRODUCTION

1.1. Cancer: an overview

1.1.1. Defining cancer

Cancer is a generic term employed to refer to a large group of diseases that can affect any part of the body. In a general way, the main defining feature of cancer is the rapid and abnormal cell growth, as well as the ability of these cells to invade other tissues and spread in the body through blood and lymphatic systems (i.e. metastasis), phenomenon which constitute the major cause of death from cancer [1].

More than 100 different types of cancer are known. Usually, cancers are termed in according to the organ or the cell type from it is originated. To simplify, cancer types can be grouped into broader categories, namely, carcinoma (begins in the skin or in tissues that cover internal organs, having several subtypes including adenocarcinoma, basal cell carcinoma, squamous cell carcinoma and transitional cell carcinoma), sarcoma (begins in bone, cartilage, fat, muscle, blood vessels or other connective or supportive tissue), leukemia (begins in blood-forming tissue, such as bone marrow , and causes abnormalities in blood cells that are produced), lymphoma and myeloma (begins in cells of the immune system) and central nervous system cancers (begin in the tissues of the brain and spinal cord) [1].

1.1.2. Cancer Statistics

Worldwide, cancer is a leading cause of death with 7.6 million deaths in 2008 and representing around 13% of all deaths [1].

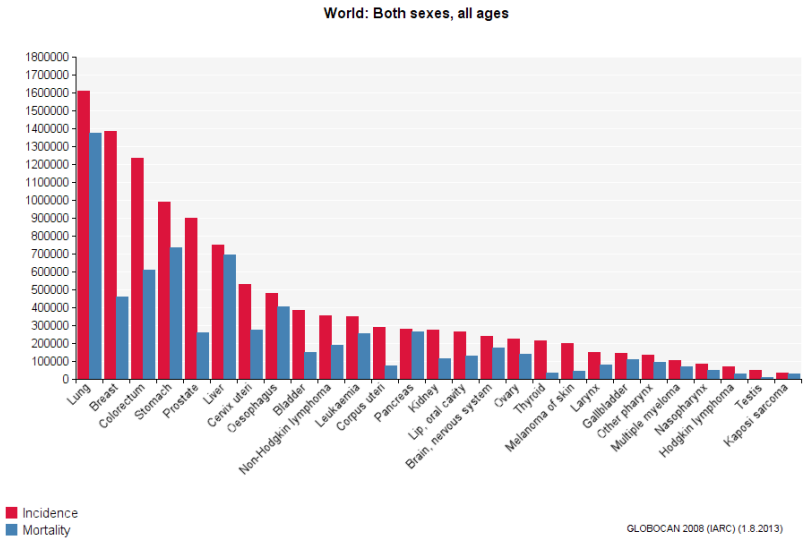


Figure 1. Worldwide incidence and mortality of cancer, in both sexes and all ages, in 2008 [1]

The most frequent types of cancer are, as shown in Figure 1, lung (1.6 million cases), breast (1.38 million cases), colorectal (1.24 million cases), stomach (1 million cases), prostate (900 000 cases) and liver (700 000 cases), while the most mortal is lung cancer (1.37 million deaths), followed by stomach and liver cancers (700 000 deaths) (see Annex I and II) [1].

Worldwide, deaths from cancer are projected to continue rising along the years, with an estimated 13.1 million deaths in 2030 representing, therefore, a serious threat to human health [1].

1.2. Prostate Cancer

1.2.1. Disease Overview

Prostate is a small gland that integrates the male reproductive system, with about the size and shape of a walnut, having a role in the production of semen (Figure 2). The prostate gland tends to grow with age, and, thereby, aging raises the risk of prostate problems, including prostate cancer [2].

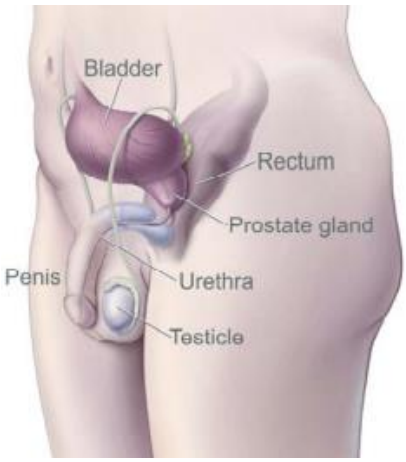


Figure 2. Prostate context in male reproductive system [2].

Prostate cancer has a slow development compared with most other types of cancer. Carcinogenesis may begin until 30 years before the tumor gets big enough to cause symptoms or be detectable and, meantime, prostate cancer cells may eventually spread throughout the body and the cancer may already be advanced. In fact, a prostate tumor may be present for several years without causing symptoms or even become a serious threat to health, until the dead for some other cause [2].

The main symptoms of prostate cancer are problems in the urine passageway, abnormal constant need to urinate, weak or interrupted urinary flow, pain or burning when passing urine, blood detection in the urine or semen, painful ejaculation. Importantly, prostate cancer can easily spread to the bones, so bone pain, especially in the back, hips or pelvis can be a symptom of advanced prostate cancer [2].

In what respect to risk factors of prostate cancer, stand out age (from 50 years-old), race (African-American men have the highest risk followed by Hispanic and Native American men, having Asian-American men the lowest rates), family history (men whose father or brothers had prostate cancer have a 2 to 3 times higher risk of prostate cancer and men who has 3 immediate family members with prostate cancer has about 10 times the risk) and diet (higher risk for men with a high-fat diet) [2].

1.2.2. The numbers of prostate cancer

Prostate cancer is the second most frequently diagnosed cancer of men (Figure 3), with over 14% of total of cancers in men (914 000 new cases) and the fifth most common cancer overall (Figure 1) [3].

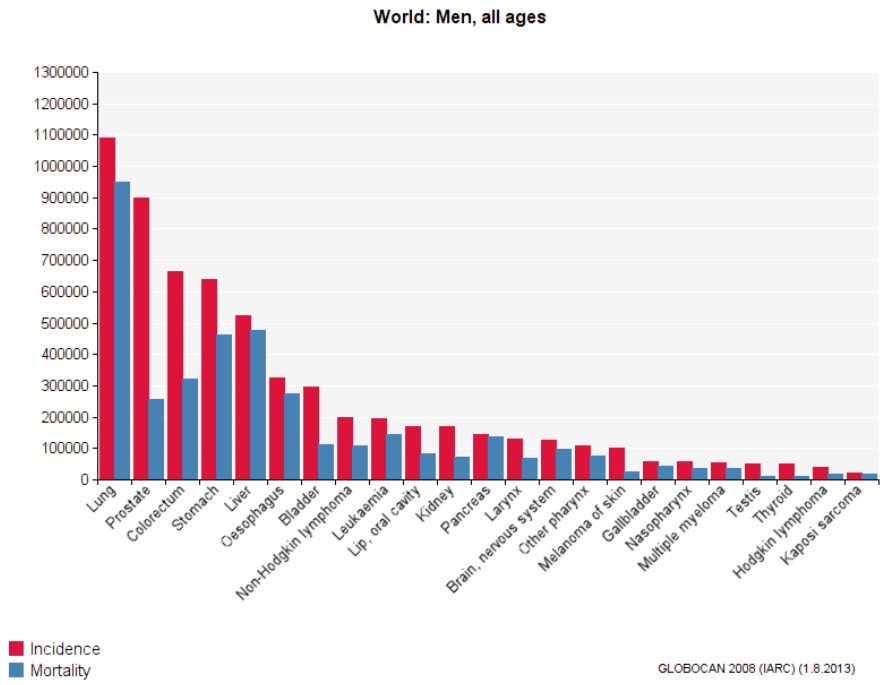


Figure 3. Worldwide incidence and mortality of prostate cancer in men, all ages, in 2008 [1].

Incidence rates of prostate cancer vary largely around the world, being the highest rates located in developed countries, like Australia/New Zealand (104.2 per 100,000), Western and Northern Europe and Northern America (Figure 4), largely because the practice of prostate specific antigen (PSA) testing and subsequent biopsy that has become widespread in those regions. Incidence rates are relatively high in certain developing regions such as the Caribbean, South America and sub-Saharan Africa, being the lowest incidence rates in South-Central Asia [3].

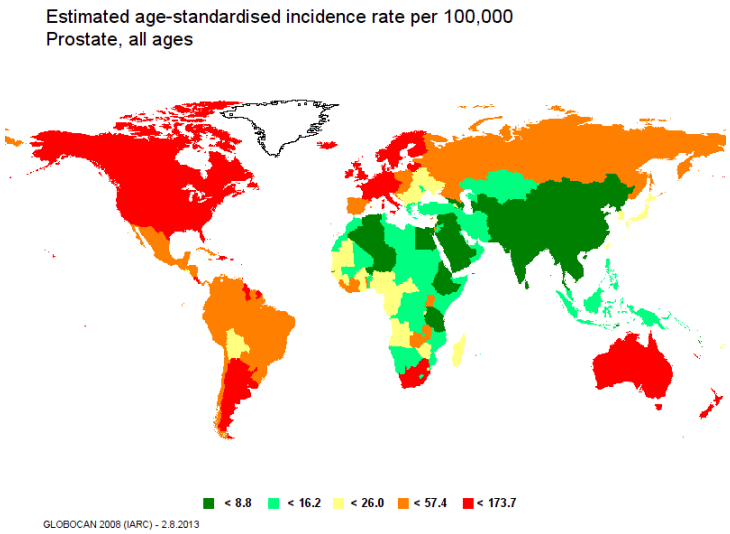


Figure 4. Worldwide incidence of prostate cancer in man, all ages, in 2008 [1].

As said before, prostate tumors have a slow development, being tightly related with the age (Figure 5) [1]. In fact, the increase in the incidence of this tumoral type in developed countries is tightly related with the increase in life expectancy, as well as with the diagnostic methods. The early diagnostic of prostate cancer possible by the prostate-specific antigen (PSA) test and the consequent successful treatment, may contribute to the observed gap between the rates of incidence and mortality rates.

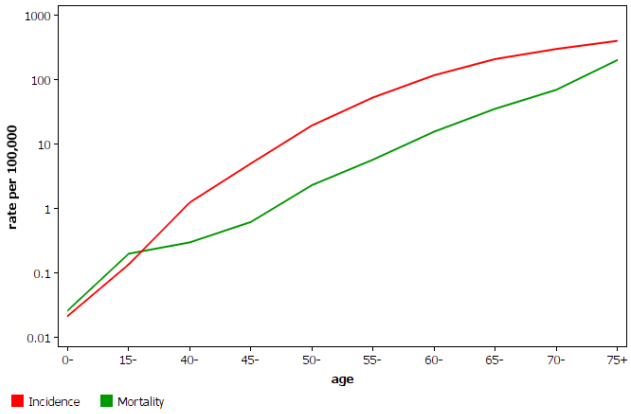


Figure 5. Incidence and mortality rates for prostate cancer according with the age [1].

1.2.3. Screening, staging and available therapies

Screening of prostate cancer is done by standard exams, being the most often used tests, after the assessment of health history and symptoms, the digital rectal exam (DRE) and the PSA test. DRE is a palpation test which permits the assessing of several characteristics such as size, firmness, and texture of the prostate, as well as the presence of hard areas, lumps, or growth spreading beyond the prostate. Pain caused by touching or pressing the prostate can also be detected. The main disadvantage is that DRE only permits the assessment of one side of prostate, not providing a global analysis of the prostate. PSA test is a U.S. Food and Drug Administration (FDA) approved test that, used along with DRE, permits the checking of prostate's health, enabling the detection of a prostate cancer. In a general way, PSA is a protein produced by prostate cells that is normally secreted by prostate, helping on semen production, and can move to the bloodstream. In case of prostate cancer, more PSA gets into the blood than is normal (>4.0 ng/mL), being a putative signal of the existence of a cancer. However, PSA test is not 100% reliable, since it can origin false-positive results, many times due to non-cancerous prostates disturbs, like prostatitis, as well as events that disturb the prostate gland like a simple bicycle riding, a prostate biopsy or surgery or even a DRE, that can increase the levels of PSA. Moreover, some prostate glands can naturally produce more PSA than others, according to the race (African-American men tend to have higher PSA levels than men of other races) and even some drugs can cause augment of the levels in the serum. For this, PSA test has to be used along with other methods of screening. This test is also often used in the follow-up of prostate cancer patients for the assessment of cancer recurrence [2].

At time of diagnosis, the extent of a cancer is a key factor to define the treatment and to assess the prognosis of the patients. The most widely used staging system among clinicians is the tumor-node-metastasis (TNM) cancer staging system (see Annex III), maintained and updated periodically by the American Joint Committee on Cancer (AJCC) and the International Union for Cancer Control (UICC), approaching important characteristics of the tumor: extent of the primary tumor (T), regional lymph nodes (N) and distant metastasis (M) [4]. Another prostate cancer classification system, widely used in North America, is the Whitmore-Jewett [5], also called ABCD rating or Jewett staging system (see Annex IV) having, however, a gap in the classification of nonpalpable tumors, detected by elevated PSA levels and consequent biopsy, not having a logical stage for these tumors, unlike the TNM system [6]. In this system, "A" and "B" refers to a localized prostate cancer, "C" refers to cancer growing outside the prostate but without spreading to lymph nodes or

other parts of the body and “D” refers to a metastatic cancer [2]. However, the most commonly used system for staging of prostate cancer is the Gleason Grading System (Figure 6) that, incorporated with other strategies, can predict the prognosis and help on therapy guiding. This grading system was proposed, in 1966, by Donald Gleason and is based on the architectural pattern of the tumor [7]. This system consisting in the sum of the two most common (up to 5% of the extent of the biopsy) patterns found in the tumor, giving a grade from 1 to 5 to each selected pattern, resulting in a score between 2 and 10, where 2 is the less aggressive and 10 is the most aggressive. [8].

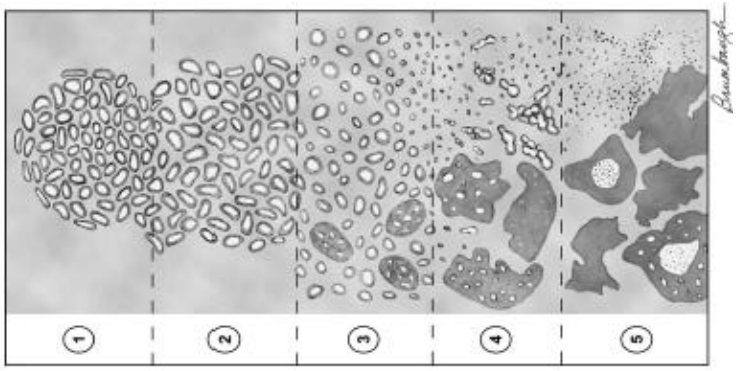


Figure 6. Schematic diagram of modified Gleason Score grading system, from the 2005 International Society of Urological Pathology Consensus Conference on Gleason Grading of prostatic carcinoma [7].

The prostate cancer treatment is dependent on cancer stage, and it is not possible to state that one therapy is clearly better than another, however, based on the randomized controlled trials and on the available literature, there are some recommendations on the most indicated and more probably successful therapeutic approach [8]. Currently, radical prostatectomy (RP) is the only effective treatment for localized prostate cancer, showing a cancer-specific survival benefit when compared to conservative management [9], being the need of pelvic lymphadenectomy controversial because of the low-risk of lymph node involvement in men. Neoadjuvant androgen deprivation, before RP, and adjuvant androgen deprivation, following RP, are controversial as the advantage on free-survival of patients is not consistent [10]. Localized prostate cancer, as well as positive surgical margins, can be treated also with radiotherapy or transperineal brachytherapy, consisting in safe and efficient techniques [11-15]. Alternatively, patients with localized prostate cancer who are not suitable for RP, can be treated by cryosurgical ablation of the prostate and high-intensity focused ultrasound [16]. Hormonal therapy, namely either complete or intermittent androgen blockade, can be also an efficient approach with proven advantage on overall survival of patients [17-19]. Chemotherapy is not a standard treatment for early prostate cancer but is

used metastatic cancers when hormone therapy is not effective [20,21]. Broad-band chemotherapeutic drugs, mainly docetaxel (the only FDA-approved), are used as last resort for this fatal stage of prostate cancer, which patients have a median survive of less than two years. However, tumors tends to develop resistance to taxanes [22,23].

Given the current landscape of available therapies against prostate cancer, as well as the invasiveness of most of them, it emerges the urgency of alternative and effective new therapies that can fill this lack.

1.3. The emergence of cancer: from normal cells to tumour

The information that allows us to understand the pathogenesis of cancer has been increasing along the years. In order to understand cancer biology it is essential to know the functional capabilities acquired by the different tumor types, during tumorigenesis [24,25]. Nowadays, it is now clear that cancer cells have distinctive and complementary capabilities giving to tumor the unlimited proliferation potential, self-sufficiency in growth signals, and resistance to antiproliferative and apoptotic stimuli [25]. Tumors are more than a mass of cancer cells, being complex tissues composed by different interacting cell types and a very special microenvironment, which is very important for carcinogenesis and contributes to the development of certain hallmark capabilities [24].

Some years ago, Hanahan and Weinberg [26] proposed the six hallmarks of cancer (Figure 7) that collectively define tumorigenesis: limitless replicative potential, self-sufficiency in proliferative signals, insensitivity to growth suppressors, evasion to cell death, sustained angiogenesis and activated invasion and metastasis capacity [24,26,27].

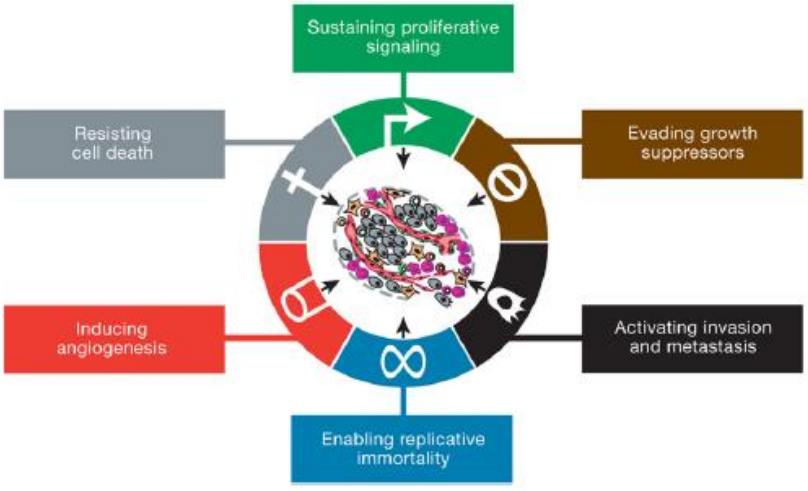


Figure 7. The six hallmarks of cancer [24]

In recent years, an increasing body of research suggested two emerging hallmarks of cancer: reprogramming of energy metabolism, consisting in a capability of cancer cells to reprogram cellular metabolism to support neoplastic proliferation in a most effective way, and evading immune system effects, having cancer cells the ability to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. At the same time, two important cancer enabling characteristics were proposed, namely the heritable molecular changes (genetic and epigenetic), that can allow hallmark capabilities, and the tumor-associated inflammation, consisting in the promotion of tumor progression by immune system (Figure 8) [24]. Recognition of these concepts is crucial to the development of new therapeutic approaches and agents that can kill tumor cells while sparing normal cells [28].

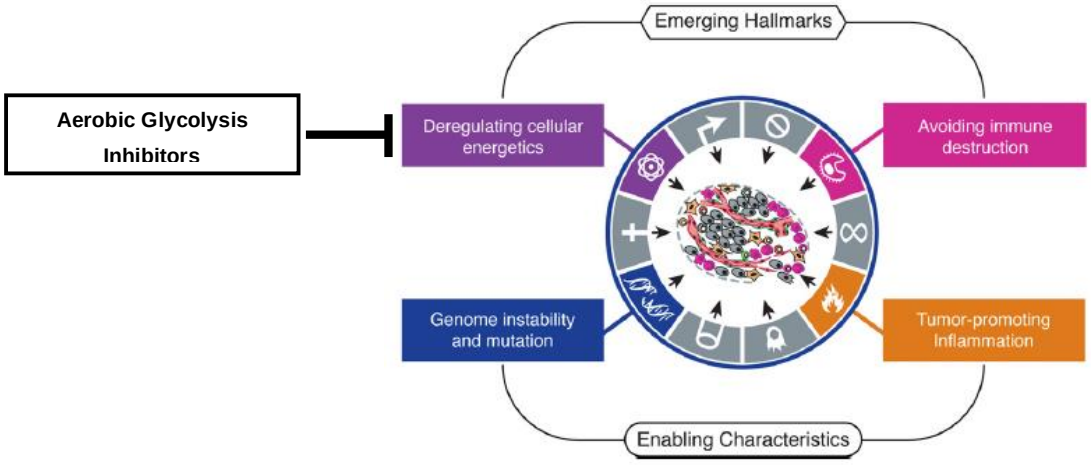


Figure 8. Emerging hallmarks of cancer, enabling characteristics and possible targeting therapy approach aiming the altered energetic metabolism of cancer cells (adapted from [24]).

1.3.1. Metabolic Reprogramming: an emerging feature of cancer

The high coordination of cellular metabolism enables the cooperation of various metabolic pathways in the conversion of nutrient molecules into other macromolecules which are required for specialized functions and to obtain chemical energy for several vital processes. In fact, cell proliferation is required for embryogenesis, growth and some functions of mammals' tissues. Differentiated tissues have basal rates of glycolysis, converting glucose to pyruvate, which is then oxidized in the tricarboxylic acid (TCA) cycle, obtaining most of the ATP (adenosine triphosphate) by a process termed as respiration or oxidative phosphorylation [29,30]. Proliferating cells can induce signals that leads to a reorganization of metabolic activity and, so, the metabolism of proliferating cells, in contrast

to quiescent cell, is characterized by high rates of glycolysis, lactate production, and biosynthesis of lipids and other macromolecules [29].

As can be seen by observing Figure 9 [31], in the presence of oxygen, nonproliferative tissues metabolize glucose to pyruvate, completely oxidize most of this pyruvate to CO_2 in the mitochondria for the process of oxidative phosphorylation, being the oxygen essential in this process as it is needed as the final electron acceptor, yielding 38 moles of ATP per molecule of glucose consumed. When oxygen is limited, cells redirect pyruvate to produce lactate, in a process called anaerobic glycolysis. This lactate production during anaerobic glycolysis allows the continuing of glycolysis, but is much less productive in terms of energy when compared with oxidative phosphorylation, only 2 moles of ATP per glucose molecule. Normal cells return to normal use of the oxidative phosphorylation when oxygen levels are restored, but in contrast, proliferative tissues and tumor cells continue to use glycolysis even in the presence of sufficient amounts of oxygen (i.e. normoxia), phenomenon known as 'aerobic glycolysis' or 'Warburg effect', an overt manifestation of modification of the tumor cells metabolism. At the same time, in the mitochondria, low rates of oxidative phosphorylation still occur in both cancer cells and proliferating normal cells. The evident lower energy efficiency of aerobic glycolysis, compared with oxidative phosphorylation, leads the tumor cells to largely increase the rates of glycolysis in order to achieve the required levels of ATP, maintaining the high proliferative rates and consequently leading the production of large quantities of lactate [31].

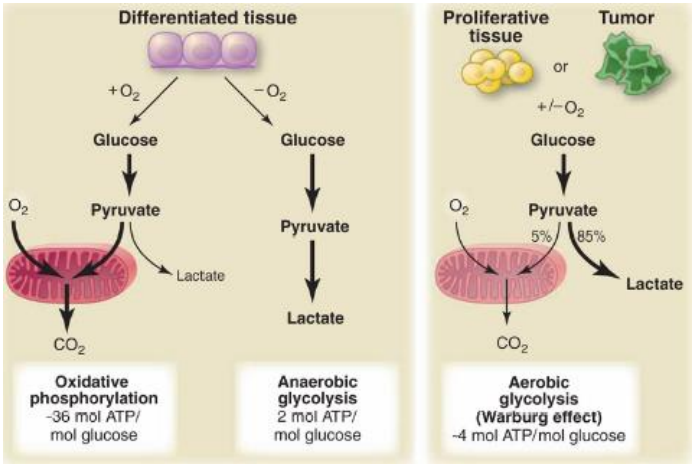


Figure 9. Schematic representation of the differences between oxidative phosphorylation, anaerobic glycolysis and aerobic glycolysis or Warburg effect [30]

Originally, Warburg described that, as result of an irreversible damage to the respiratory chain (oxidative phosphorylation), increased glycolysis is one way to

compensate for the failed ordinary energy-producing energy. [32] Currently, it is known that the metabolic changes that occur during carcinogenesis results from genetic modifications that may include activation of proto-oncogenes and inactivation of tumor suppressor genes [31]. However, although Warburg's original hypothesis has proven incorrect, the original observation of increased glycolysis in tumors has been repeatedly confirmed and the increasing on glucose uptake has, in fact, proved useful for tumour detection and monitoring. ^{18}F-fluorodeoxyglucose positron emission tomography (FdG-PET) image technique (Figure 10) has as basis the hyperglycolytic phenotype of cancer and has been largely used in clinical context for diagnosis, staging, prediction and monitoring of treatment response and surveillance of patients from various types of cancers [33,34]. The main principle of this technique relies on the activity of glucose transporters that are responsible for the uptake of the glucose analogue 2-(18F)-fluoro-2-deoxy-D-glucose (FDG), which after phosphorylation, is no further metabolized and becomes trapped in the cells. Accumulation of the radioactive glucose analogue is then detected by PET, allowing to detect tumours in the body [35,36].

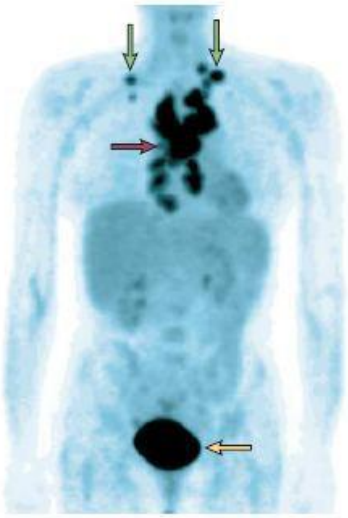


Figure 10. PET imaging with FDG of a patient with lymphoma. The nodes represented by purple and green arrows show a high uptake of FDG, showing that tumors in these nodes have high levels of FDG uptake. The bladder (yellow arrow) also has a high activity, because of excretion of the radionuclide [31].

Although the glycolytic phenotype is less efficient compared to oxidative phosphorylation, it is the quickest way of ATP production, suggesting that cancer cells have increased metabolic autonomy [31,37,38]. Tumour cells also can divert intermediates of the glycolytic pathway to anabolic reactions, allowing the biosynthesis of lipids, amino acids and also nucleotides [31,38-41]. Importantly, the Warburg effect induces a decrease in reactive oxygen species (ROS) production, protecting cells from oxidative stress. Additionally, the

greater production of lactic acid (lactate), which contributes to acidic microenvironment, confers advantages to tumour cell growth and proliferation, increasing several malignant features like migration, invasion and metastisation [37,38].

During carcinogenesis, aerobic glycolysis can cooperate with mitochondria during cancer progression, evidencing the metabolic plasticity of cancer cells [41-44]. In fact, recent studies demonstrate a directly link between Warburg effect and mitochondrial defects, as well as to oncogenic activation and mutation in signaling pathways that regulate glucose uptake, such as PI3K/Akt/mTOR pathway [37,40]. Additionally, selection of the glycolytic phenotype in tumour cells can be mediated by the action of transcription factors [34,38,45], like the hypoxia-inducible factor 1-alpha (HIF-1α) [45], MYC [45], Ras and p53 [46].

Thus, a nearly universal property of cancer, both primary and metastatic, is increased glycolysis, resulting in increased consumption of glucose leading, consequently, to a hyperglycolytic phenotype. This hyperglycolytic phenotype leads to microenvironmental acidification requiring changes in cell phenotype in order to resist to acid-induced cell toxicity. The following cell populations with increased glycolysis and acid resistance, have a powerful growth advantage, which promotes a free proliferation and invasion [31].

1.3.2. Tumoral microenvironment: acidic and hypoxic

The avascular environment where carcinogenesis have risen, presupposes a dependence, by cancer cells, on glucose and oxygen diffusion, through blood vessels and basement membrane, in order to achieve their major metabolic needs [31] and, in fact, early carcinogenesis cannot occur more than a few cell layers far from the basement membrane or regional hypoxia would limit cell growth. Thus, tumor cells have to perform several adaptations on their metabolism, like rapid ATP production, increased biosynthesis of macromolecules and maintenance of adequate cellular redox status [47], facing the stressful and dynamic microenvironment of solid tumors, where concentrations of some nutrients like glucose, glutamine and even oxygen are spatially and temporally heterogeneous [48]. Moreover, the intermittent hypoxia conditions observed in tumor microenvironment leads to a Darwinian-like selective pressure on cancer cells, contributing to the constitutive up-regulation of glycolytic phenotype observed in most tumors [31,37,47,49]. Additionally, characteristics of tumors like the abnormal vasculature and the altered pH can influence tumour microenvironment, contributing also to the glycolytic phenotype [50].

Then, the tumour cell population that emerges from the referred evolutionary sequence, acquiring a constitutive glycolytic phenotype by selective pressure of hypoxia,

has a powerful proliferative advantage, being able to alter their environment in a way that is harmless to itself, but toxic to other cellular phenotypes. The environmental acidosis, through increased concentration of lactate, contributes to the invasive phenotype through degradation of the extracellular matrix and promotion of angiogenesis (Figure 11) [31].

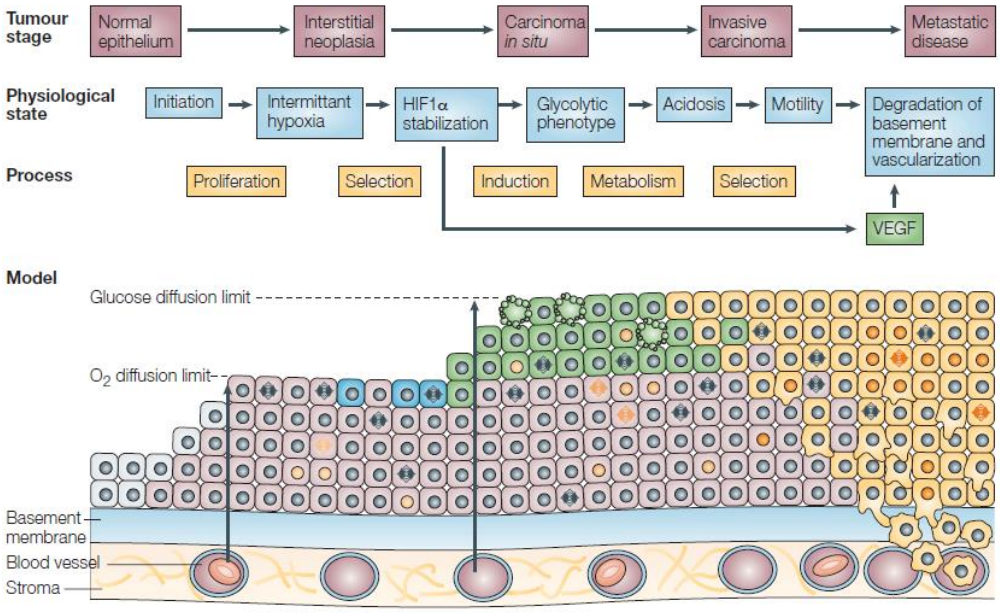


Figure 11. Model for cell-environment interactions in carcinogenesis [31]. Cell colours represent different cell types: grey for normal epithelial cells, pink for hyper-proliferative cells, blue for hypoxic cells, green for glycolytic cells and yellow for motile cells. Light orange nuclei represent one mutation while dark orange nuclei represent more than one mutation. Blebbing membranes show apoptotic cells. Abbreviations: HIF-1α, hypoxiainducible factor 1 alpha; VEGF, vascular endothelial growth factor.

1.3.2.1. Hypoxia: a “only for a few” favorable environment

Hypoxia is a common microenvironmental feature of malignancy in solid tumors. Altogether, several factors contribute to deficient oxygen supply (i. e. hypoxia) in tumor cells, namely the inability to keep angiogenesis with tumour growth, the presence of disorganized and abnormal vasculature within the tumour and also the increased hydrostatic pressure within the tumour leading to microvasculature compression [41]. As response to an adverse environment, tumour cells do not induce cell death but otherwise initiate response mechanisms that favors cell survival and migration, namely activation of the transcriptional factor HIF-1α, a widely known regulator of hypoxic stress, which has been widely associated with cancer progression [51-56]. This transcription factor is a heterodimer composed of an oxygen dependent α subunit and a constitutively expressed non-oxygen dependent β subunit. HIF-1 α subunit is degraded in the presence of oxygen (> 5% O_2) but otherwise is stabilized under hypoxic conditions (< 5%O_2) and translocated to the nucleus

forming a HIF-α/β complex which can bind to target genes on their hypoxic-response elements (HREs) [57,58]. The binding of HIF-α/β to HREs results in transcriptional up-regulation of target genes that mediate multiple adaptations that leads to resistance to hypoxia, namely enzymes involved in the glycolytic pathway, as well as the vascular endothelial growth factor (VEGF) that promotes angiogenesis (Figure 12) [59].

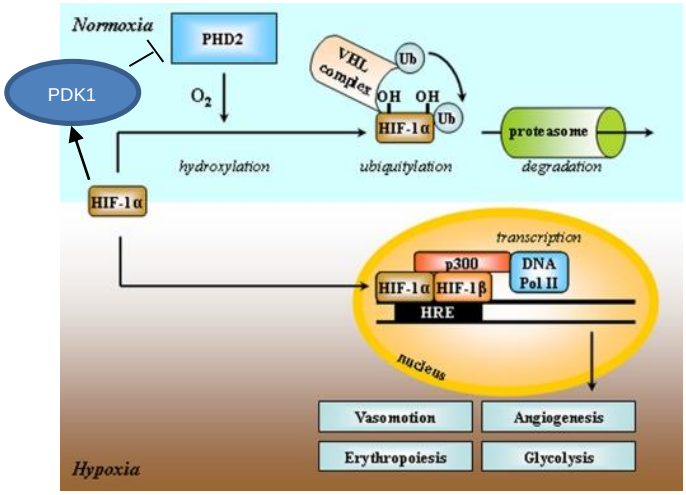


Figure 12. Scheme representative of HIF-1α degradation under normoxia conditions, and activation by hypoxia (adapted from [66]). Under normoxia conditions, HIF-1α is hydroxylated, ubiquitylated and further degraded. Under hypoxia conditions, HIF-1α protein is expressed, migrates to the nucleus and binds to HIF-β and further to HRE in promoting regions, which induce glycolysis, angiogenesis, erythropoiesis and vasomotion.

In fact, as one may think, the up-regulation of HIF-1α in various types of solid tumors is associated with poor clinical outcome [59]. Importantly, HIF-1α activation is effective, not only in stimulating glycolysis, but also in decreasing oxidative phosphorylation, which allows to equilibrate O_2 consumption with the poor O_2 supply [60]. In a general way, HIF-1α can compromise oxygen consumption in oxidative phosphorylation by induction of pyruvate dehydrogenase kinase-1 (PDK1) expression, which inhibits the mitochondrial pyruvate dehydrogenase (PDH), preventing conversion of pyruvate into acetyl-CoA and consequently its diversion to the respiratory pathway (Figure 13). One more time, as consequence of global oxidative phosphorylation reduction, hypoxic tumour cells are forced to increase the glycolytic metabolism in order to maintain the production of ATP for cell survival. Therefore, HIF-1α regulates the expression of glucose transporters (GLUT-1 and GLUT-3) to increase glucose uptake and also induces the expression of several downstream glycolytic, such as hexokinase II (HK2) and pyruvate kinase type 2 (PKM2). The metabolic reprogramming mediated by HIF-1α redirects pyruvate to another HIF-1α target, lactate dehydrogenase (LDH-A) that catalyzes the conversion of pyruvate to lactate [58,60-62]. The increase in

lactic acid production amongst the diminished vascular dispersion of CO_2 contributes to hypoxic acidosis, and therefore to the acid-resistant phenotype. In this context, other type of proteins, that are also targeted by HIF-1α, arises for pH regulation, namely carbonic anhydrase IX (CAIX), which is a hypoxic marker and a prognostic indicator which performs the reversible conversion of CO_2 to bicarbonate and proton, contributing to extracellular acidification of tumour microenvironment and consequently to control of intracellular pH [58,63], and monocarboxylate transporters (MCTs) [57,58,61]. It is already described the up-regulation of MCT4 in hypoxia, where it cooperates with MCT1 in lactate efflux [58,64] and, for that, MCTs will have a dual role in cancer, both for the acid-resistant phenotype and for the hyper-glycolytic phenotype, allowing the continuous conversion of pyruvate to lactate by performing the lactate efflux and consequent clearance of the glycolytic pathway [65].

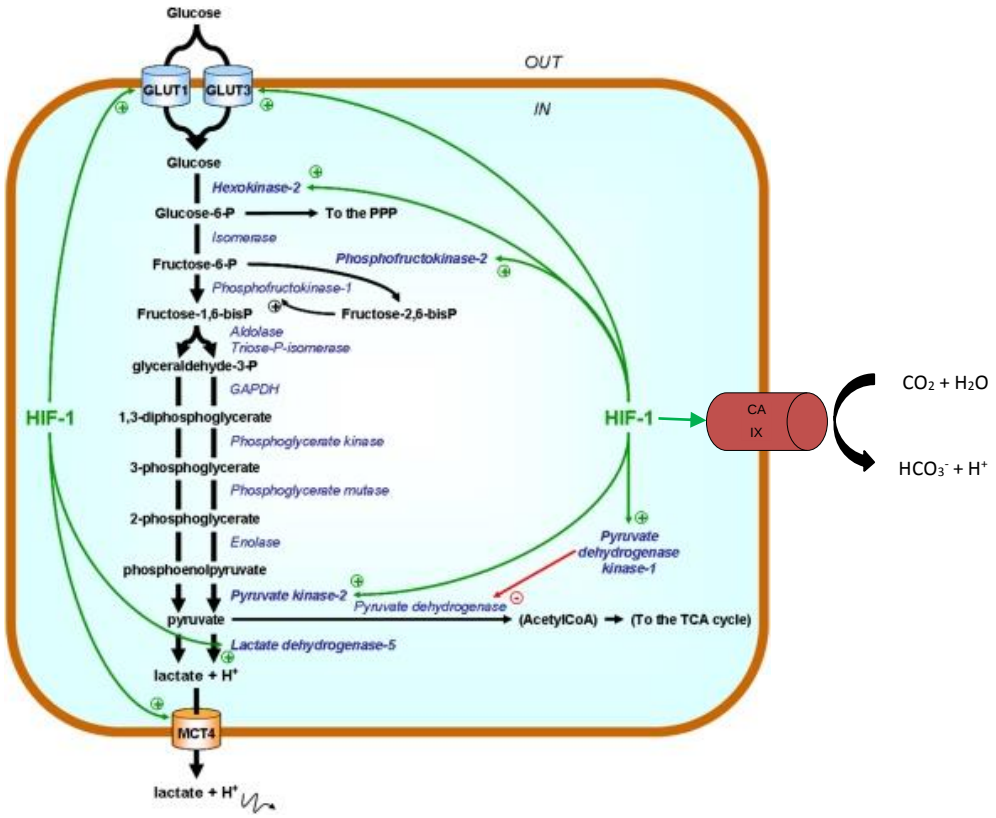


Figure 13. HIF-1 promotes the expression of glycolytic enzymes and transporters (adapted from [66]). Green arrows point at HIF-1 target gene products directly involved in increased glycolytic flux. Abbreviations: CAIX: carbonic anhydrase IX; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; MCT4, monocarboxylate transporter 4;PPP, pentose phosphate pathway; TCA, tricarboxylic acid (cycle).

The frequency and severity of tumor hypoxia and its association with malignant progression make hypoxia induced metabolic changes promising therapeutic targets for cancer therapy [47] and, in fact, some clinical trials are already in course [66].

1.3.2.2. Lactate transport: a great contributor to malignancy

The increase in glycolysis typical in cancer cells leads to acidification of tumour microenvironment, having cancer cells, as referred above, different membrane pH regulatory mechanisms. This include the activity of proteins, such as MCTs, CAIX, as well as Na^+/H^+ exchanger 1 (NHE1) and anion exchanger 1 (AE1), that allow the survival, growing and selection of cancer cells in this microenvironment which otherwise will be fatal. Although MCTs are not the major proton transporters in cells, its importance turns out to be enhanced due to their dual role in the adaptation to the microenvironment: export of lactate - essential to the hyper-glycolytic phenotype - and pH regulation, important to the acidic-resistant phenotype [67]. The acidification of the extracellular space is, in a large part, mediated by MCTs, specifically MCT1 and MCT4, due to the co-transport of lactate with protons [31]. It is known that microenvironmental acidity is associated with cancer cell invasion and migration, allowing metastasis [68,69] and that lactate contributes to several features of tumour progression and malignancy, like immune escape [70], angiogenesis [68,71], radioresistance [72] and high incidence of distant metastasis [73], highlighting its active role, and therefore the role of MCTs, in progression of malignant diseases (Figure 14) [69,73].

Lactate produced by glycolytic tumour cells is largely associated with poor prognosis, disease-free and overall survival in several cancers like cervical cancer [74], head and neck cancer [75], high grade gliomas [76], non-small-cell lung metastasis [73] and prostate [77], as well as with radioresistance in some tumors caused, in part, by the antioxidant properties of lactate [69]. Additionally, evidence shows that, independently from the oxygen conditions, lactate can regulate hypoxia inducible genes by stimulating the accumulation of HIF-1α [78], which would lead, in turn, to a stimulation of the glycolytic pathway by regulating glycolytic enzymes as already referred, providing an important positive feedback in the context of cancer. Moreover, lactate was demonstrated to increase cellular motility [79], VEGF [80], as well as molecules involved in the process of cancer invasion and metastisation, namely hyaluronan and its receptor CD44 [81,82] and even the expression of proteins associated with increased migration capacity of cancer cells, namely transforming growth factor (TGF-β2) [83]. Importantly, besides the glycolysis, there are other pathways that, in a lower extent, can contribute to lactate production in solid tumors, such as glutaminolysis and serinolysis (Figure 14) [84,85].

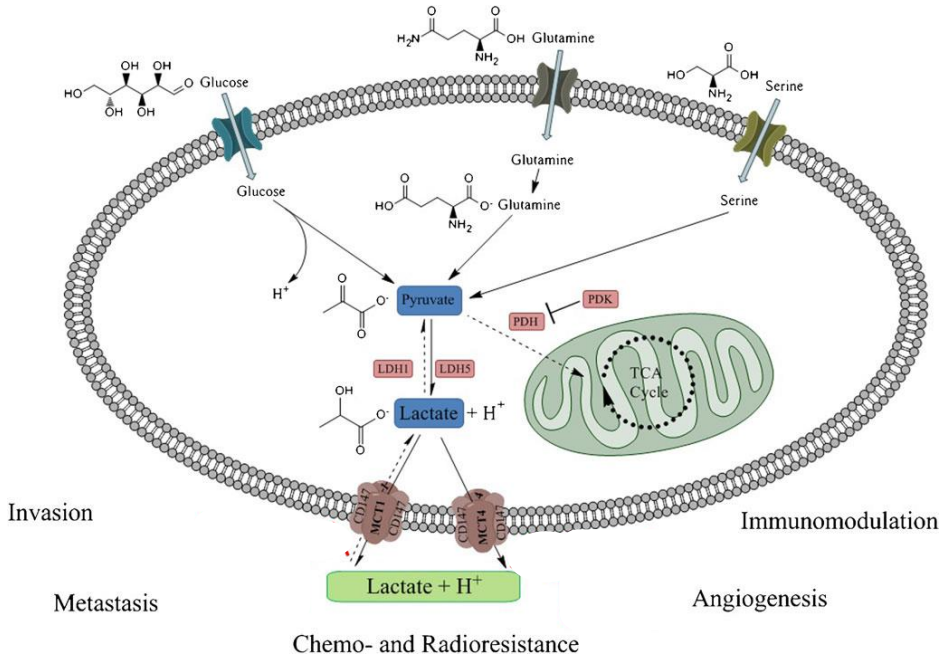


Figure 14. Overview on the metabolic pathways leading to lactate production (continuous lines) and transport across the plasma membrane (adapted from [67]. Discontinuous arrows represent lactate uptake and flow inside oxidative cancer cells. Abbreviations: LDH, lactate dehydrogenase; MCT, monocarboxylate transporter; PDH, pyruvate dehydrogenase; PDK1, pyruvate dehydrogenase kinase 1.

However, lactate properties that contribute to malignancy do not stop here. Besides its referred roles in acidification, mutation, promotion of invasive behavior and factor in radioresistance of cancer cells [31], lactate has another important role, not least important, in the tumoral aggressiveness context, namely on the T-cell activation, which is dependent on high rates of glycolysis and on the rapid efflux of lactate from T cells [86], but stay compromised if the extracellular concentrations of lactate are sufficiently high leading to a negative feedback that blocks its efflux and thus disturbs the metabolism and function of T cells, consequently decreasing the immune response against tumour cells [70].

Moreover, besides being mainly an end-product, lactate exported from a cell may also be a substrate for neighbor cells, as described in skeletal muscle and brain [87-89], phenomenon currently known as the “cell-cell lactate shuttle”, in which the peripheral and oxygenated oxidative cells consume the lactate produced by the central and hypoxic glycolytic cells (Figure 15). Additionally, intracellular lactate has been also described as a key metabolic intermediate in a symbiosis between glycolytic and oxidative cancer cells, by entering the mitochondria via MCTs [65,90].

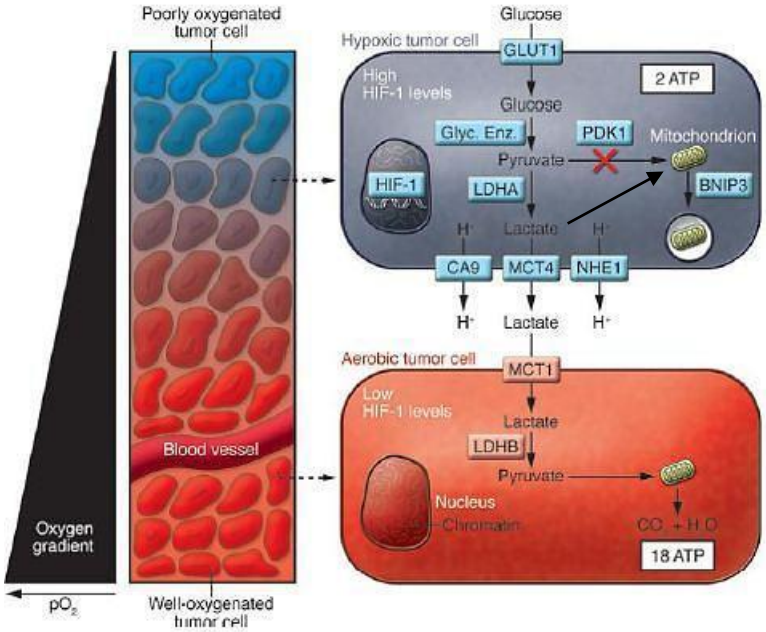


Figure 15. Intratumoral hypoxia and metabolic symbiosis. LDHA/B, lactate dehydrogenase A/B; PDK1, pyruvate dehydrogenase kinase1; MCT1/4, monocarboxylate transporters 1/ 4; CAIX, carbonic anhydrase IX; NHE1, Sodium-Hydrogen Exchanger1; HIF-1α, hypoxia-inducible factor (adapted from [90]).

Altogether, these characteristics and roles allow lactate to be seen as a suitable candidate for diagnostic and prognostic indicator for a wide variety of tumors, being reasonable to think that targeting of its transport may be a promising approach for cancer therapy [68].

1.4. Monocarboxylate Transporters

Monocarboxylic acids, such as lactate and pyruvate, play a key role in cellular metabolism and in inter-tissue communication. [91] The rapid transport of these compounds across the plasma membrane is essential for this role, being mediated by the monocarboxylate transporters (MCTs), as shown in Figure 16 [91].

The presence of MCTs is vital in tissues which energy supply relies on glycolysis and therefore depends on the intracellular lactate transport across the plasma membrane to maintain the high glycolytic rates, such as white skeletal muscle, red blood cells and tumour cells. Additionally, tissues like brain, heart and red skeletal muscle, without forgetting gluconeogenic tissues, like kidney and liver tissues, where lactate could be the major source of energy, being oxidized as a respiratory fuel, are also dependent on MCTs' activity in the uptake of lactate into the cells [91].

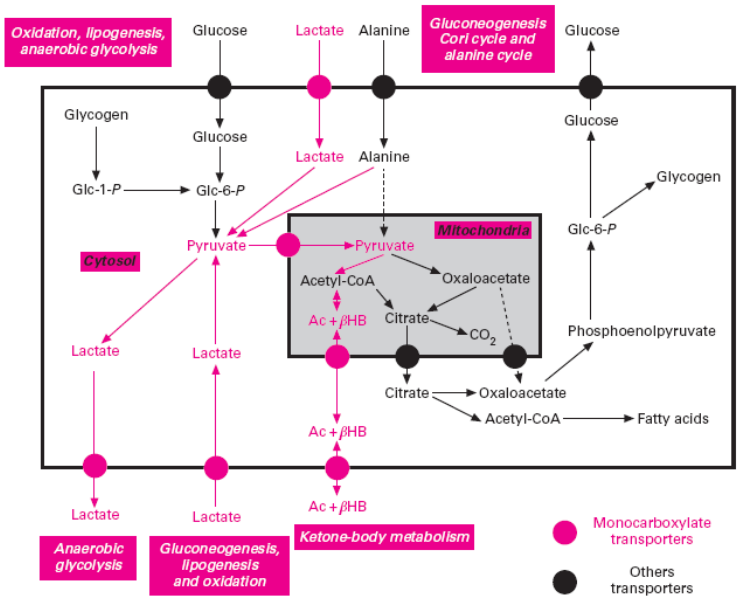


Figure 16. Diagram of the metabolic pathways involving the monocarboxylate transporter through the plasma membrane and mitochondria [91]. Abbreviations: Glc-1-*P* and Glc-6-*P*, glucose 1-phosphate and glucose 6-phosphate; Ac-bHB, acetoacetate plus b-hydroxybutyrate.

In fact, MCTs have a central role in mammalian cellular metabolism as well as in the communication between cells since they catalyze the lactate facilitated transport across the plasma membrane in a proton symport way, being upregulated in cancer cells, which have increased glycolysis. [91] Although lactate is the main monocarboxylic acid transported by MCTs, there are also other metabolically important monocarboxylates transported such as pyruvate, branched-chain oxo-acids derived from leucine, valine and isoleucine, acetoacetate, β-hydroxybutyrate and ethyl acetate [91]. The bet in the characterization of monocarboxylate transport in different cell types like erythrocytes, cardiac myocytes and hepatocytes, among others, led to the hypothesis of the existence of a MCT family [92].

1.4.1. The MCT family

The Solute Carrier Family 16 (SLC16), or MCT family, is encoded by the *SLC16* genes, showing conservation among species including mouse, rat and chicken [93]. Phylogenetic studies allowed the identification of the 14 known members of MCT family (*SLC16* genes), and provided important information about the functional clustering of the human MCT family [67]. Figure 17 shows that MCT1-MCT4 are associated in the same cluster, presenting a high homology and, in fact, after functional characterization, only MCT1-MCT4 have demonstrated the proton-linked transport of monocarboxylic acids [67].

Importantly, this cluster is yet sub-divided into two branches, MCT1-2 and MCT3-4, which correlate with the transported substrate as well as the affinities by which those transport is performed [94-101].

The topological prediction of MCTs show a structure of 12 transmembrane domain (TMD) helices with both intracellular amino and carboxyl terminal, presenting a large loop between TMDs 6 and 7 and having a range of 29-105 amino acid residues [102]. These transmembrane regions are the most conserved [103], in contrast to the hydrophilic regions, which show little conservation avoiding those regions to be the responsible for the transport and possibly pointing their role to functional aspects such as substrate specificity or regulation of activity [104].

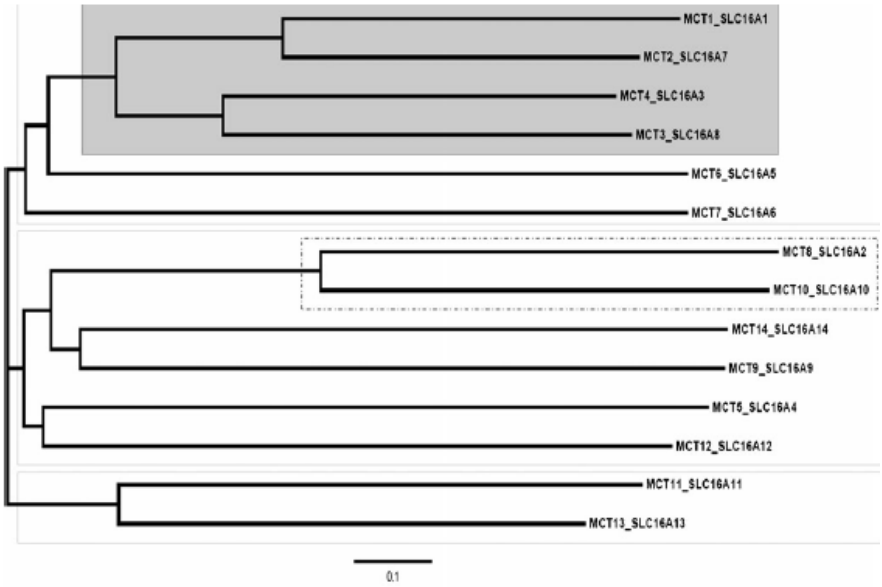


Figure 17. Human MCT family members' phylogram, based on amino acid sequence. Boxes limited by dots represent three main clusters. In the doted-dashed box are the thyroid hormone (MCT8) and aromatic amino acids (MCT10) transporters. Solid grey box represent the proton-linked transported cluster (MCT1-4) [67].

Topological prediction for MCT1 was experimentally confirmed in erythrocytes (Figure 18) [105] but a similar topology could be predicted for other isoforms based on aminoacidic sequence analysis [93]. In what regards proton coupling, typical from MCTs, the N-terminal domain of the MCT protein is pointed to be the main responsible, being also associated with the membrane insertion and the maintenance of the structure. On the other hand, C-terminal domain is pointed to be important for substrate affinity [91]. The ability of MCTs to transport monocarboxylates, along with a proton, across the plasma membrane lead to the reasonable prediction that TMD's amino acids are more likely responsible for proton binding

and translocation being the only conserved aspartate/glutamate residue in the N-terminal already described as a candidate [91,93,106]. In fact, studies of site-directed mutagenesis in highly conserved residues of MCTs, confirmed the role of TMDs on lactate transport carried out by this protein [91,103]. Additionally, the presence of asparagine's in extracellular loops of the transporter would denote possible sites for glycosylation, a common phenomenon for membrane spanning proteins, despite the theoretical predictions and experimental evidence indicates absence of glycosylation in MCTs [107].

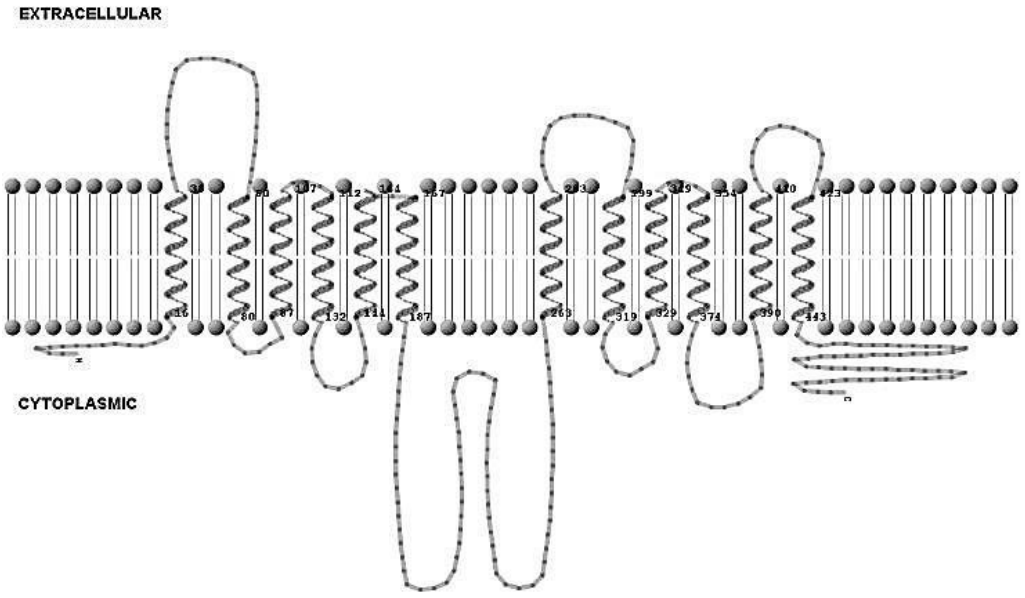


Figure 18. MCT1 protein diagram [105].

1.4.1.1. MCT isoforms

The main differences between the various MCTs isoforms are related to substrate and inhibitor affinities, the regulation of their expression, and their tissue distribution and intracellular localization [108]. Here, it will be focus mainly on MCT1, MCT2 and MCT4 isoforms, whose function is responsible for the name of the family of transporters and more relevant in cancer.

1.4.1.1.1. MCT1

SLC16A1, the human MCT1 gene (NCBI Reference Sequence: NP_001159968.1), was cloned by Garcia and colleagues in 1994, in Chinese hamster ovary cells (CHO) [109] and subsequently, by others, in human, rat and mouse [102], constituting the most studied MCT isoform, due to both its unique expression in human erythrocytes and its ubiquitous

tissue distribution [107]. Although it was already known that *SLC16A1* was located in chromosome 1, the structural gene characterization of *SLC16A1* promoter was only achieved in 2002 [110] bringing these results the information that the transcription of MCT1 have a universal promoter, which is consistent with its broad tissue distribution [107]. As functional protein, MCT1 is composed by 494 amino acids, having a molecular weight of ~54 kDa [106].

As already referred, MCT1 is present in several and different cells and tissues, such as in blood-brain barrier (BBB), T-lymphocytes, spermatogenic cells, brain, apical membrane of retina pigmented epithelium (RPE), inner ear, kidney, stomach, liver, gut epithelium, among others, where it is required for membrane transport of monocarboxylates [107]. Additionally, MCT1 has been described to be expressed in mitochondria [111] and peroxisomes [112] where it seems to participate in a lactate oxidation complex to maintain organelle redox status.

In what regards the transport activity, MCT1 exhibits an intermediate substrate affinity, allowing it to perform both the uptake and efflux of monocarboxylates from cells, which may constituting an explanation for its wide pattern of expression [67]. Importantly, MCT1 is capable to transport a large variety of substrates, including short chain (C2-C5) unbranched aliphatic monocarboxylates such as acetate and propionate, as well as monocarboxylates with C2 or C3 substitutions, as pyruvate, L-lactate, β-hydroxybutyrate and acetoacetate and more poorly, formate and D-lactate [67,91,93,103,107]. Bicarbonate, dicarboxylates, tricarboxylates and sulphonates cannot be transported [93] and may even act as potent competitive inhibitors of the transport of other monocarboxylates [103].

Importantly, the MCT1 optimal plasma membrane expression and function on export or uptake of monocarboxylates requires a chaperone, CD147 (described later), as shown in Figure 19 [93].

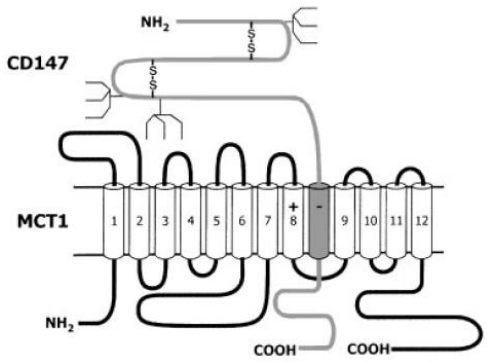


Figure 19. Membrane topology proposed for MCT1 and CD147 [93].

1.4.1.1.2. MCT2

SLC16A7, firstly cloned and sequenced from a hamster liver cDNA library by Golstein and Brown's group and, more recently, characterized in *Xenopus* oocytes, has already been cloned and sequenced from rat, mouse and human, being located in chromosome 12, containing 484 amino acids and having a molecular weight of ~52kDa [91].

The properties of MCT2 (NCBI Reference Sequence: NP_004722.2) are not well understood in comparison with other isoforms of MCTs. MCT2 shows limited distribution, in contrast to MCT1, existing evidences for alternative spliced mRNA species in human and rat [104,113]. This isoform shares with MCT1 about 60% of its sequence however, several analyses have been shown that MCT2 has a much more restricted tissue distribution and expression level when compared to MCT1 [91,93,106-108]. High levels of MCT2 expression was found in human testis and colon [102,107] and moderate to low levels in spleen, heart, kidney, pancreas, skeletal muscle, brain, and leukocyte [107]. In tissues expressing both MCT2 and MCT1, the expression differs in location, suggesting distinct functional roles between these isoforms which may be related to high substrate affinity verified for MCT2 [103], in contrast to MCT1. Importantly, MCT2 also catalyzes the proton-linked transport of a variety of monocarboxylates, however, a preference was demonstrated for both pyruvate transport and L-lactate [106,107] in human MCT2 expressed in *Xenopus* oocytes [107], as well as for ketone bodies and β- hydroxybutyrate [106]. Therefore, MCT2 appears as a high affinity transporter, performing preferentially the uptake of monocarboxylates into the cells in normal cell metabolism [99], being consequently found in tissues that use lactate as a respiratory fuel, like brain or cardiac and skeletal muscle, as well as gluconeogenic tissues where lactate is the major substrate, like kidney and liver [104]. Additionally, as well as MCT1, MCT2 is also found in mitochondria [114]. For its optimal membrane expression and function, MCT2 needs also a chaperone, called gp-70 or EMBIGIN [113].

1.4.1.1.3. MCT4

MCT4 isoform (NCBI Reference Sequence: NP_001035887.1), encoded by *SLC16A3* gene, was initially called MCT3 based on its sequence homology with the MCT3 described in chicken, identified by Price and colleagues. Lately, when a distinct MCT3 was identified in mammalian retinal pigment epithelium (RPE), the name was modified for MCT4 [91,103]. The human MCT4, is located in chromosome 17, being constituted by 465 amino acids and corresponding to a molecular weight of ~50kDa.

In opposition to MCT2, and as well as MCT1, MCT4 has a wide distribution, being described its particular strong expression, and therefore having particular importance, in glycolytic tissues which need to export lactate, such as white skeletal muscle fibers, astrocytes, white blood cells and chondrocytes and, as it could not fail to be, in tumors [93,103,108]. Consistently, MCT4 appears in exclusively neonatal heart, which is more glycolytic than adult heart as well as in placenta, being involved in the transference of lactate into the maternal circulation [93]. Altogether, this facts indicates that the main activity of MCT4 may be the lactate efflux derived from glycolysis [103] which is consistent with its kinetic properties [100], which shows a lower affinity for L-lactate and pyruvate than MCT1 and MCT2 [93,102,103,107]. Such as MCT1, MCT4 requires CD147 as a chaperone [91].

1.4.1.1.4. Other MCT isoforms

Besides MCT1 (*SLC16A1*), MCT2 (*SLC16A7*) and MCT4 (*SLC16A3*), there are other isoforms of MCTs associated with different anatomical features, which have been characterized along the years [91]. Firstly, MCT3 is preferentially expressed in the base-lateral membrane of the RPE and has a role in regulation of pH and lactate concentrations in the outer retina [106,107,115], choroid plexus [106,107] and brain [116]. MCT6 (*SLC16A5*) is a bumetanide transporter, which carriage is very sensitive to pH and membrane potential, without dependence on the proton gradient [93,117]. MCT8 (*SLC16A2*) is described as a thyroid hormone transporter [118], recently in parallel with *SLC18A10* gene which also encodes for an aromatic amino-acid transporter (T-type amino-acid transporter 1), being known as TAT1 more than MCT10 [91,103]. *SLC16A2* is a thyroid hormone transporter and mutations in this gene have been associated with X linked severe mental retardation and neurological dysfunction [103]. MCT12 (*SLC16A12*) has the known function of setting/maintaining homeostasis in the cells of the eye and kidney [119] and, although its substrate is still unknown, is already described that mutations in this gene are associated with development of cataracts [93]. Importantly, *SLC16A12* has been identified as a possible biomarker for colon, prostate and breast carcinoma, due to gene hypermethylation [120]. MCT9 is associated with changes in uric acid [93,121], however its specific action and substrate remains unknown, like for other members of the family (MCT5, MCT7, MCT11, and MCT13 MCT14).

1.4.2. MCT regulation by chaperones

As previously mentioned, functional expression of MCTs depends on a type of accessory proteins for their trafficking and anchoring to the plasma membrane, called chaperones, since MCTs are not glycosylated, a common characteristic of membrane proteins [67,91,93,106,107]. Presently, the described chaperones for MCT1 and MCT4, major glycolysis-associated carriers, and therefore with the greater relevance in cancer, are both CD147 and CD44.

CD147, also known as basigin (BSG), EMMPRIN (Extracellular matrix metalloproteinase inducer), OX-47 or HT7, is a membrane glycoprotein widely distributed in the plasma membrane and belongs to the immunoglobulin superfamily (Ig) [122]. One of the main functions of CD147 is to induce the production of matrix metalloproteinases that degrade extracellular matrix, promoting invasion and metastasis of tumor cells [122]. Specifically interacts with MCT1, MCT3 and MCT4, but not with MCT2, playing an essential role in their functions [123] and therefore it becomes important in the metabolism of tumor cells of several cancers [123-131]. Moreover, the co-expression of CD147 with MCT1 is associated with poor prognostic features, supporting the action of CD147 as chaperone and matrix metalloproteinase inducer, as observed in breast and gastric carcinoma [128,130]. Besides the role of CD147 as chaperone for MCT1 and MCT4 plasma membrane trafficking and activity, these MCT isoforms have, in turn, implications in CD147 membrane expression [124,125], so the contribution of MCTs to the malignant phenotype is not only at the level of its function on lactate transport and pH regulators, but also in their role on regulation of CD147 expression, which can enhance several malignant behaviors of cancer cells, like migration and invasion [132-135]. Therefore, targeting CD147, which also impair MCT activity, appears to be a rational therapeutic approach against human cancer, as already described both *in vitro* and *in vivo* [136-138].

CD44, in turn, is a multistructural and multifunctional cell surface molecule involved in cell proliferation, cell differentiation, cell migration, angiogenesis, presentation of cytokines, chemokines, and growth factors to the corresponding receptors, and docking of proteases at the cell membrane, as well as in signaling for cell survival. All these biological properties are essential to the physiological activities of normal cells, but they are also associated with the pathologic activities of cancer cells [139]. CD44 may also function as a chaperone for MCT expression [135], being described to be associated with MCT1 in lung cancer [129]. Thus, the interaction with CD44, enhances the role of MCTs in cell growth control, adhesion, migration, invasion and chemo-resistance [140-142]. However, the prognostic value of this

protein has been shown inconsistencies as it is associated both with both poor and favorable prognosis, depending on the tumor type, so these doubts have to be overcome before the application of targeted therapies against CD44 in human cancers [139].

Additionally, there are studies already describing an interaction of embigin, known MCT2 chaperone, with MCT1 in rat erythrocytes [143], however further studies are needed to investigate this putative interaction in the context of cancer.

Importantly, there is a relevant number of cases with MCT plasma membrane expression with a lack of CD147 or CD44 co-expression, suggesting that more chaperones, not yet identified, may be involved in MCT function [67].

1.5. MCT expression in human solid tumors

Recapitulating, high levels of lactate production and the consequent microenvironmental acidosis, is a common feature observed in tumour cells, leading to the assumption that MCTs, especially MCT1 and MCT4, which are the major transporters of lactate across plasma membrane, may have a central role in cancer [103,108]. In the last years, studies reported upregulation of MCTs in different human solid tumors, showing evidence on importance of MCTs in cancer biology.

Evidence for MCT up-regulation was found in colorectal carcinomas [129,144-146], lung cancer [129,147], soft tissue sarcomas [148], cervical cancer [127,149], ovarian cancer [150], pancreatic cancer [136], prostate cancer [77,151], breast carcinomas [130,152], gastrointestinal stromal tumors (GISTs) [153], glioblastoma [154] and even melanoma [155]. Lambert and colleagues described, some years ago, that the transition area from normal to malignant phenotype of a tumor, presented a down-regulation in MCT1 expression [146], however, more recent studies shows evidence for the upregulation of MCTs in colon adenocarcinoma [129], pairing with a significant increase of MCT expression in cancer cells in comparison with normal cells [144,145]. This contradictions, may have arisen at the level of antibody specificity, which is consistent with the lack of membrane expression for MCT1 in the first study, which would be imperative for the correct role of MCTs in lactate export [67]. Importantly, association of MCT expression with clinicpathological data of patients showed significant associations between membrane expression of MCTs and vascular invasion [144].

Epithelial ovarian cancer, both primary and metastatic, shows an up-regulation of MCT1 and MCT4 in about 80%, compared with normal and benign ovarian tissues [150].

Pinheiro and colleagues, also reported expression of MCT1, MCT2 and MCT4 in ovarian carcinoma, but with a lower frequency for MCT4 [129].

An association was found in cervical cancer between lactate production, and therefore the role of MCTs, and the increased incidence of metastasis and short disease free and overall survival [149]. Pinheiro *et al.* found a significant increase in MCT1 and MCT4 expression both from pre-invasive to invasive squamous lesions and from normal glandular epithelium to adenocarcinomas [149].

In gastrointestinal stromal tumors (GISTs), MCT1, MCT2 and MCT4 are highly expressed, being the co-expression of MCT1 and CD147 associated with low overall survival of the patients [153].

Ependymomas, hemangioblastomas and high grade gliomas (anaplastic astrocytomas and glioblastoma multiforme) show an upregulation of MCT1 in comparison with the general absence of its expression in low-grade glial neoplasms (oligodendrogliomas and astrocytomas) [154,156].

In lung cancer, MCT1 show overexpression in the plasma membrane, in contrast with normal lung where there was no expression of this MCT isoform [157]. However, a study by Pinheiro and colleagues showed a high frequency of MCT1 expression in normal lung, being MCT4 less expressed in tumoral than in normal lung [129]. Additionally, a cytoplasmic expression of MCT1 was described in alveolar soft tissue sarcoma [148].

Evidence for MCT down-regulation was described in breast cancer [152]. However, a study of Pinheiro and colleagues demonstrated a significant increase of MCT1 either in cytoplasm or plasma of breast carcinoma cells, in comparison with normal breast epithelium, being MCT4 expression more cytoplasmic [129,130]. Importantly, MCT1 expression shows an association with basal-like subtype of breast cancer [130].

In gastric carcinoma, the results were different of those obtained for the already referred tumor types. In fact, MCT1 was similarly expressed in normal gastric mucosa, primary tumors and lymph-node metastasis, which may indicates the role of MCT1 in gastric homeostasis maintenance, both in normal tissue and along carcinogenesis. Moreover, MCT4 expression showed a significant decrease in the plasma membrane of gastric cancer cells [128].

Finally, in what regards prostate carcinoma, the available studies are controversial [151,158] and more studies are warranted to better elucidate the expression pattern of MCT in prostate tissues. The available information on MCT expression in prostate cancer will be developed in the next section.

Altogether, the available data suggests the contribution of MCTs to the hyper-glycolytic and acid-resistant phenotype, being the up-regulation of MCTs in the plasma membrane of the different types of tumors, an adaptive mechanism to allow continuous high glycolytic rates, by exporting the accumulating end-product, lactate, as well as to counteract the acid-induced apoptosis or necrosis [67].

1.5.1. Expression of MCTs in prostate cancer

As said in the previous section, knowledge on the role of MCTs in prostate cancer is somehow controversial, highlighting the need of more studies in this field.

Hao *et al.*, in a first approach, described MCT1 and MCT4 as being widely expressed in prostate cancer and unexpressed in normal prostate tissues [151]. However, Pértega-Gomes and colleagues, showed an increase in MCT2 and MCT4 isoforms, while MCT1 appeared to have a decreased expression in human samples of prostate cancer, when compared with normal tissues [158]. Importantly, MCTs showed to be expressed preferentially between normal and tumoral tissue in PIN lesions, suggesting the role of these proteins in the malignant transformation. It was also found an association between MCT1, MCT4 and CD147 expression and poor prognosis of the patients, being MCT4 and CD147 overexpression correlated with higher PSA levels, Gleason Score, pT stage, perineural invasion and biochemical recurrence [77]. Pértega-Gomes *et al.* also demonstrate that, along with α-methylacyl-CoA racemase (AMACR), a marker already used in prostate cancer diagnosis [77,159], MCT2 is consistently expressed in prostate cancer, becoming a putative positive biomarker in prostate cancer, highly enhancing the accuracy of prostate cancer diagnosis [160] when used along with the two known negative markers, p63 and 34βE12 [160].

1.6. MCT inhibition: a promising weapon against cancer

Carcinogenesis is the reflex of several genetic alterations which leads, among other effects, to a reorganization of metabolic pathways to support biosynthesis, allowing the cell autonomous nutrient uptake and therefore enhancing cell resistance to death [39,48]. This metabolic alterations, along with the already referred hypoxia-induced metabolic alterations, and its association with malignant progression constitute promising targets for cancer therapy [47]. In fact, the development of treatments that target tumour metabolism has been

a bet of research in the last years, with several potential drugs for metabolic pathways-targeting, being already in clinical trial phase (Figure 20) [28,66,161].

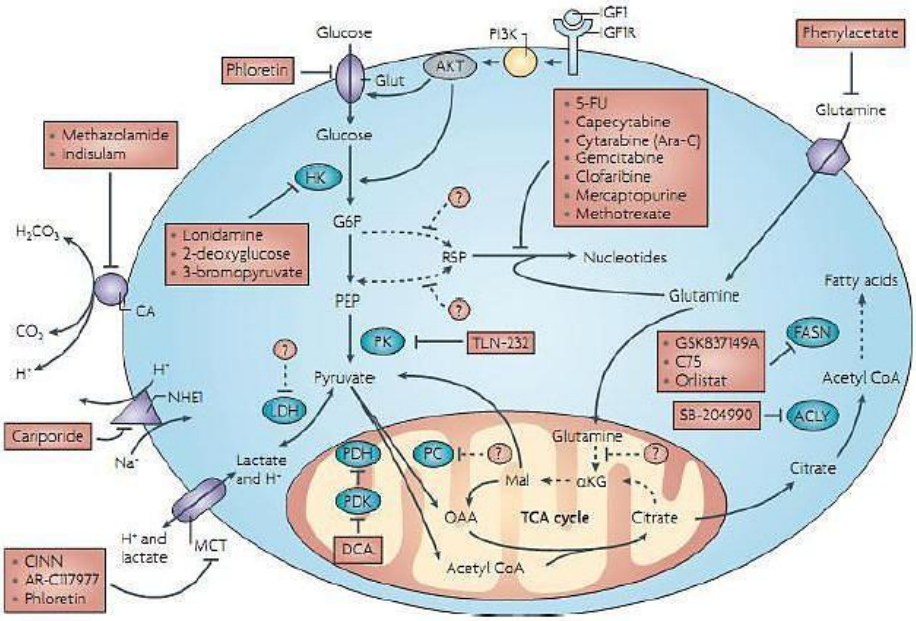


Figure 20. Actual and future therapeutic targets (dashed lines) of tumour metabolism by targeting metabolic enzymes [160]. 5-FU, 5-fluorouracil; αKG, α-ketoglutarate; ACLY, ATP citrate lyase; CA, carbonic anhydrase; CINN, α-cyano-4-hydroxycinnamate; DCA, dichloroacetate; FASN, fatty acid synthase; G6P, glucose-6- phosphate; Glut, glucose transporter; HK, hexokinase; IGF1, insulin-like growth factor 1; IGF1R, IGF1 receptor; LDH, lactate dehydrogenase; Mal, malate; MCT, monocarboxylate transporter; NHE1, Sodium-Hydrogen Exchanger 1; OAA, oxaloacetate; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; PEP, phosphoenol pyruvate; PK, pyruvate kinase; R5P, ribose 5-phosphate; TCA, tricarboxylic acid cycle.

As already mentioned, the acid-resistant phenotype is essential for cancer cell survival, being MCTs important pH regulators that contribute to that phenotype [162]. Although MCTs are not the major H^+ transporters, they perform a dual role in cancer adaptation to microenvironment: export of lactate, essential for the maintenance of the hyper-glycolytic phenotype, and pH regulation, important for the acid-resistant phenotype. Accordingly, MCT inhibition will have a direct effect on cell pH regulation and glycolytic rates, therefore having an important effect on cell viability [67]. Additionally, MCTs have a crucial role in metabolic symbiosis between cancer cells [65], so targeting these transporters will “shut-down” the advantageous symbiosis, likely having an important impact on tumour homeostasis. Finally, taking into account the contribution of lactate to the malignant phenotype, together with the up-regulation of MCTs in some tumors, MCT inhibition may be a useful therapeutic approach in cancer. This will contribute to increase the immune response against tumour cells and decreased its migration capacity, among others [67]. Also MCT inhibition, namely MCT1, offers the opportunity to simultaneously target tumour metabolism and angiogenesis [65,66,71].

In the last years, it was demonstrated that *in vitro* MCT1 inhibition decreases intracellular pH [65,155,163], leads to cell death [65,163-165] and, importantly, enhances cancer cell radiosensivity [165]. Additionally, MCT4 silencing shows effectiveness in decreasing migration capacity of cancer cells [124], by mechanisms that involve interaction of MCT4 with β-integrin [166]. Silencing of MCT1 and MCT4 inhibits cancer cell invasion [157]. Moreover, studies with the MCT1 inhibitor α-cyano-4-hydroxycinnamate (CHC) showed promising results once it demonstrated capacity for lag tumor growth and increase tumor radiosensivity [65], decreasing tumour invasion [165]. Also, lonidamine [163] and small interference (siRNA) have been used to inhibit the activity or expression of MCTs, respectively, showing relevant effects on tumor [65,163,164]. Also, the relevance of MCTs for tumour growth was confirmed by a more specific approach, where combined silencing of MCT1, MCT4 and CD147 significantly decreased glycolytic rates and retarded tumour growth [167]. Importantly, the use of MCT1 specific inhibitors, already designed by AstraZeneca, may also be an effective strategy to block MCT1 activity in cancer [168], having a related orally administered compound, AZD3965 (AstraZeneca), currently in Phase I/II clinical trials for treating advanced solid tumours [66].

1.6.1. MCT targeting in prostate cancer

Little is known about the true effects that may result from blocking the function of MCTs in prostate cancer. In theory, as well as by experience in other types of cancer, MCT targeting may be a promising therapeutic approach against prostate cancer, in general, and against the aggressiveness phenotype and angiogenesis in particular.

An *in vivo* study from Kim and colleagues, showed that MCT1 activity inhibition using CHC did not have a significant effect on tumour volume, although it was associated with increased necrotic fraction [169].

Since there are yet no sufficient reports about the true relevance of MCTs in the development and progression of prostate cancer, it is important to invest in studies assessing both the blocking and silencing of MCTs in this type of cancer, in order to reach a conclusion about the effectiveness of this therapeutic approach.

CHAPTER 2: AIMS

2.1. Rationale and aims

Currently, there is a lack of effective therapies against prostate cancer, being the existent therapies much invasive for patients. Thus, once prostate cancer is one of the most incident cancers worldwide in men, it becomes urgent to perform studies exploring new therapies in this type of cancer.

In this work, we propose the approach of MCTs as targets for a new targeted therapy in prostate cancer. As previously mentioned, MCTs play an important role in the maintenance of glycolytic metabolism, by exporting lactate by a proton symport mechanism. Importantly, a previous study performed by our group [158] showed that MCT1 and the chaperone CD147 are downregulated in human prostate cancer tissues. Moreover, the same study described the presence of MCT4 in the transition area from normal to tumoral tissues, suggesting its role in prostate cancer progression, being the presence of both MCT1 and MCT4 along with CD147 associated with poor prognosis of patients [158].

Thus, laying the described for cases of human prostate cancer where MCTs are associated with prostate cancer progression, as well as with poor prognosis of patients, becomes relevant bet in the study of the real contribution of MCTs to the survival and aggressiveness of prostate cancer in order to assess their potential as a therapeutic target in this type of cancer.

So, the specific aims of this project are:

1. Characterize the expression of MCTs and chaperones, as well as the expression of selected metabolic marker relevant in glycolysis, such as GLUT1, CAIX, HKII, LDH, PDK, PDH and HIF-1α. in different prostate cell lines;
2. Determine the effect of MCT1 and MCT4 downregulation, by siRNA, in prostate cancer, by assessment of important parameters in cancer such as cell viability, proliferation and migration, using *in vitro* models of prostate cancer;
3. Evaluate the contribution of MCTs to prostate tumour growth and angiogenesis by performing the downregulation of MCTs in an *in vivo* model, namely chicken chorioallantoic membrane (CAM) model.

CHAPTER 3: MATERIAL AND METHODS

3.1. Cell Culture

The *in vitro* experiments were performed using different prostate cell lines as models, all obtained from the American Type Culture Collection (ATCC). 22RV1 (ATCC® CRL-2505™) cell line is derived from a localized human prostate tumor and DU145 (ATCC® HTB-81™) is derived from a metastatic prostate tumor on brain, so as to draw conclusions according to tumor aggressiveness (localized or metastatic).

Cell lines were maintained in Roswell Park Memorial Institute-1640 medium (RPMI, GIBCO, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco, Invitrogen) and 1% of antibiotic (penicillin/streptomycin, 10 µg/ml, Gibco, Invitrogen), at 37ºC in a humidified atmosphere of 5% CO2.

3.2. Preparation of Cell-Paraffin-Blocks

For protein characterization and localization, cells were grown in T75 flasks, in RPMI with 10% FBS and 5% Penicillin-Streptomycin (Gibco, Invitrogen), until 95% confluence. Then, cells were trypsinized and centrifuged at 1200 rpm, for 5 minutes. Cell pellets were fixed in formaldehyde 3.7%, overnight and re-centrifuged and then processed in an automatic tissue processor (TP1020; Leica), before inclusion into paraffin-blocks (block-forming unit EG1140H; Leica).

3.3. Immunocytochemistry

Immunocytochemistry (ICC) for MCT1, MCT4, CD147, CD44, GLUT-1, CAIX, HKII, LDH5, PDK, PDH and HIF-1alpha was performed in 4µm cytoblock sections, from the previously made cell-paraffin-blocks and staining was analyzed for protein expression, distinguishing cytoplasmic from plasma membrane expression. ICC is a technique based on the principle of antibody-antigen interaction that allows the detection of target proteins in biological tissues, providing a direct method to identifying the subcellular protein distribution. The antibody-antigen interaction is visualized using a chromogen, in which the enzyme conjugated to the antibody cleaves a substrate to produce a colour precipitate (brown colour) at the location of the interest protein. IHC for MCT1, PDK, PDH and HIF-1α was performed according with the avidin-biotin-peroxidase principle (R.T.U. Vectastin Elite ABC kit; Vector Laboratories). For CD147, CD44, MCT4, GLUT-1, CAIX, LDH and HKII, IHC was performed with the Ultravision Detection System Anti-polyvalent, horseradish peroxidase (HRP) (Lab

Vision Corporation). Briefly, deparaffinised and rehydrated slides were submitted to adequate heat-induced antigen retrieval, for 20 minutes, at 98ºC, followed by endogenous peroxidase activity inactivation. After the blocking step of 10 minutes (LabVision kit) or 20 minutes (Vector kit) slides were incubated with the primary antibody. After, rising in phosphate-buffer saline (PBS), slides were incubated with secondary biotinylated antibody, for 10 minutes (LabVision kit) or 30 minutes (Vector Kit), and then incubated with streptavidin peroxidase for 10 minutes (LabVision kit) or avidin peroxidase for 45 minutes (Vector kit) at 37ºC. The immune reaction was visualized with 3-3'-Diaminobenzidine (DAB+ Substrate System; Dako) as a chromogen. All sections were counterstained with Gill-2 haematoxylin and mounted in Entellan®. Each immunoreaction was performed according to the specified in the Table I.

Table I. Immunocytochemistry details for each antibody.

Antibody	Company	Reference	Dilution	Antigen retrieval	Peroxidase inactivation	Positive control
MCT1	Santa Cruz Biotechnology	sc-365501	1 :500	EDTA (1mM, pH=8)	3% H202 in methanol, 30min	Colon carcinoma
MCT2	Santa Cruz Biotechnology	sc-14926	1 :200	Citrate (10mM, pH=6)	3% H202 in methanol, 10min	Colon carcinoma
MCT4	Santa Cruz Biotechnology	sc-50329	1 :500	Citrate (10mM, pH=6)	3% H202 in methanol, 10min	Colon carcinoma
CD147	Santa Cruz Biotechnology	sc-71038	1 :400	EDTA (1mM, pH=8)	3% H202 in methanol, 10min	Colon carcinoma
CD44	AbDSerotec	MCA2726	1 :1000	Citrate (10mM, pH=6)	3% H202 in methanol, 10min	Colon carcinoma
GLUT-1	Abcam	ab15309-500	1 :500	Citrate (10mM, pH=6)	3% H202 in methanol, 10min	Colon carcinoma
CAIX	Abcam	ab15086	1 :2000	Citrate (10mM, pH=6)	3% H202 in methanol, 10min	Stomach
LDH-5	Abcam	ab101562	1 :1000	EDTA (1mM, pH=8)	3% H202 in methanol, 10min	Colon
PDH	Abcam	ab67592	1 :300	EDTA (1mM, pH=8)	3% H202 in methanol, 10min	Stomach
PDK	Abcam	ab110025	1 :500	EDTA (1mM, pH=8)	3% H202 in methanol, 10min	Stomach
HKII	Abcam	ab104836	1 :750	EDTA (1mM, pH=8)	3% H202 in methanol, 10min	Colon carcinoma
HIF-1α	BD Bioscience	610958	1 :100	EDTA (1mM, pH=8)	3% H202 in methanol, 10min	Glioblastoma

3.4. Western blotting

The Western blot is an analytical technique used to detect specific proteins in sample extracts. Gel electrophoresis is used to separate denatured proteins and the proteins are transferred into a membrane, being labeled with specific antibodies to target protein(s). Cells were grown to 80% confluence, in T25 flasks and then protein samples were prepared by collecting the cells in lysis buffer containing 1% Triton-X, 1% NP-40, 0,1 mM EDTA, 50 mM Tris pH 7.5, 150 mM NaCl and 1/7 protease inhibitor cocktail, for 15 minutes and then centrifuged at 13.000 rpm, 15 minutes, at 4ºC. The supernatant was collected and the protein concentrations determined according to the Bio-Rad Dc Protein Assay (500-0113, Bio Rad). Aliquots of 20 µl of total protein were separated on 10 % (w/v) polyacrylamide gels by SDS-PAGE and transferred into nitrocellulose membranes (Hybond-c Extra, Amersham Bioscience) using a wet system. Membranes were blocked with 5% milk, in TBS/TW 0.1% Tween for 1 hour at room temperature. After overnight incubation at 4ºC with the primary polyclonal antibodies, membranes were washed in TBS/0.1% Tween and incubated with the respective secondary antibodies. Signals of the bound antibodies were detected by chemiluminescence (Supersignal West Femto kit, Pierce, Thermo Scientific). β-Actin was used as the positive control. Table II shows the specifications needed for each antibody.

Table II. Western blotting details for each antibody.

Antibody	Company	Reference	Dilution	Secondary Antibody Species
MCT1	Santa Cruz Biotechnology	sc-365501	1 :500 in 5% milk	Anti-Mouse
MCT2	Santa Cruz Biotechnology	sc-14926	1 :200 in 5% milk	Anti-Rabbit
MCT4	Santa Cruz Biotechnology	sc-50329	1 :2000 in 5% milk	Anti-Rabbit
CD147	Santa Cruz Biotechnology	sc-71038	1 :200 in 5% milk	Anti-Mouse
CD44	AbDSerotec	MCA2726	1:500 in 5% milk	Anti-Mouse
GLUT-1	Abcam	ab15309-500	1:800 in 5% BSA	Anti-Rabbit
CAIX	Abcam	ab15086	1:1000 in 5% milk	Anti-Rabbit
LDH-5	Abcam	ab101562	1:2000 in 5% milk	Anti-Rabbit
PDH	Abcam	ab67592	1:300 in 5% milk	Anti-Mouse
PDK	Abcam	ab110025	1:2000 in 5% milk	Anti-Mouse
HKII	Abcam	ab104836	1:2000 in 5% milk	Anti-Mouse
HIF-1α	BD Bioscience	610958	1:1000 in 5% BSA	Anti-Mouse
β-Actin	Santa Cruz Biotechnology	sc-1616	1:300 in 5% milk	Anti-Goat

3.5. Small-interference RNA and Lipofection

Small interfering RNA (siRNA) is a class of double-stranded RNA molecules, with 20-25 base pairs in length which can interfere with the expression of specific genes with complementary nucleotide sequence and can be introduced into the cells by means

of Lipofection technique, which uses liposomes, or vesicles, that can easily merge with the cell membrane since they are both made of a phospholipid bilayer and release compounds into the cell [170,171]. The silencing mixture contained Opti-MEM® (Minimal Essential Medium, Gibco), siRNA (Santa Cruz Biotechnologies) for each gene under studied and a control (scramble) for each one, and Lipofectamine® RNAi MAX Transfection Reagent (Invitrogen), added last, in order to allow the siRNA to be enclosed into the vesicles formed. The needed quantities are described in Table 3. After 15 min of incubation, 500 ml of the mixture were placed into separate wells, in 6-well plates. Cells growing in T25 flasks were trypsinized, resuspended in RPMI with 10% FBS and without antibiotic, since the antibiotic could be detrimental to cell survival due to the stress caused by lipofection. The density of the cell suspension was determined using the Trypan Blue test, by counting in a Neubauer chamber. 22RV1 cells were plated at a density of 500,000 cells per well and DU145 at a density of 400,000 cells per well, in a total of 1500 ml per well. The following day, the culture medium was changed to RPMI with 10% FBS and 1% antibiotic. After some optimizations, it was found that silencing was maximum, in both cell lines, on the 4th day of silencing and remains unchanged until the 6th day after silencing.

Table III. Quantities (in µL) of needed reagents for the mixture to silence MCTs in cells by the lipofection technique.

	Condition	Optimem	siRNA	Lipofectamine
22RV1	***scramble***	500	5	3
	siMCT1	500	5	3
DU145	***scramble***	500	7	3
	siMCT1+4	500	2 (MCT1)+5 (MCT4)	3

3.6. Cell Viability Assay

Cell viability was analyzed using the Sulforhodamine B assay (SRB, TOX-6, Sigma-Aldrich). The SRB assay is a colorimetric assay based on the ability of the dye Sulforhodamine B to bind electrostatically and pH dependent to protein basic amino acid residues of trichloroacetic acidfixed cells. Under mild acidic conditions it binds to the cells and under mild basic conditions it can be extracted from cells and solubilized for measurement. An increase and/or decrease in the cell number (total biomass) results in a simultaneous change in the amount of dye incorporated by the cells in the culture, which indirectly indicates the degree of cytotoxicity caused by the knockdown of MCTs. Cell lines were plated into 96-well plates, at a density of 9000 cells per well for 22RV1 and 8000 cells for DU145 and allowed to adhere overnight in complete medium. The effect of the

knockdown of MCT1 and MCT4 on cell number (total biomass) was determined at 12 and 24 hours. After reaching the specific time points, the cell culture medium was aspirated and the wells were washed with PBS 1x. Then, 100 µl of cold TCA 10% were added per well and the plate was incubated during 1 hour at 4 ºC. Following incubation, the wells were washed three times with water and allowed to air dry thoroughly (at least 24 hours). When the wells were dried, 50 µl of Sulforhodamine B were added and the plate was incubated for 30 minutes at room temperature. When the incubation was over, wells were quickly rinsed four times with acetic acid 1%, and allowed to dry during 30 minutes until no moisture was visible. To finalize the assay, 100 µl of Tris 10 mM were added per well and, in a shaker, the plate was allowed to incubate for 5 minutes. The absorbance was read at 490 nm, with a background absorbance of 655 nm. The results were analyzed using Graph Pad Software version 5.03.

3.7. Cell Proliferation Assay

The effect of MCTs knockdown on cell proliferation was assessed using the 5-bromo-2´-deoxyuridine (BrdU) assay which is a precise, fast, and simple colorimetric assay to measure cell proliferation, which is based on the measurement of BrdU incorporation during DNA synthesis in replicating cells. 22RV1 and DU145 were plated in 96-well culture plates, at a cell density of 8000 and 9000 per well, respectively. Cells were allowed to adhere overnight. The medium was changed to RPMI medium (without FBS). The plates were allowed to incubate for 18 hours at 37 ºC in a humidified atmosphere of 5% CO2 and 5 µl of 5-bromo-2´-deoxyuridine (BrdU, 400µM, Roche Applied Science) were added per well. Cells were re-incubated for an additional 6 hours (total of 24 hours of treatment). Following incubation, the labeling medium was removed by suction and 100 µl/well of FixDenat was added to the cells. The cells were incubated for 30 minutes at room temperature. The FixDenat solution was removed and 100 µl/well of anti-BrdU POD working solution (1:100) were added. After 90 minutes incubation, the antibody conjugate was removed and the wells were washed five times with 200-300 µl of PBS 1x. Finally, the washing solution was removed and 100 µl/well of substrate solution were added. After 5-30 minutes incubation, the reactions were stopped using 25 µl of 1M H2SO4 (Sigma) and the absorbance was measured in a micro-plate reader (Infinit M200, Tecan) at 450 nm with a reference wavelength of 690 nm.

3.8. Metabolism assays

Metabolism of prostate cell lines was assessed by glucose and lactate quantification using two colorimetric assays. For that, 22RV1 and DU145 cells were seeded in 48-well plates (Nunc, Thermo Scientific), at a density of 500.000 and 400.000 cells per well, respectively, and allowed to adhere overnight, in complete medium (RPMI, 10% FBS, 5% antibiotics). The medium was changed to RPMI (without FBS) at T_0. After 12 and 24 hours, 100 µl of supernatant were collected from each well and transferred to a 96-well plate (Nunc, Thermo Scientific) for glucose (Roche Applied Science) and lactate (Spinreact) quantification.

3.8.1. Extracellular Glucose Measurement

In this colorimetric assay, the enzyme glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid and hydrogen peroxide (H_2O_2). The H_2O_2 is detected by a chromogenic oxygen acceptor, phenol, 4-aminophenazone (4-AP) in the presence of peroxidase (POD). The intensity of the color formed is proportional to the glucose concentration in the sample.

$$\text{B-D-Glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

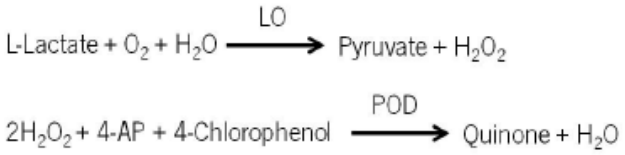
$$H_2O_2 + \text{Phenol} + \text{4-AP} \xrightarrow{\text{POD}} \text{Quinone} + H_2O$$

In a 96-well plate, 10 µl of supernatant were diluted in 90 µl PBS 1x (1:10). After that, 10 µl of the first dilution were diluted in 90 µl of PBS 1x (total dilution 1:100). A calibration curve based in a range of glucose solutions with different concentrations was performed. Finally, 100 µl of glucose reagent was added to the wells and the plate was incubated at room temperature for 20 minutes. The blank was made with 100 µl PBS and 100 µl of glucose reagent. Absorbance was read at 490 nm.

3.8.2. Extracellular Lactate Measurement

In this colorimetric assay, the enzyme lactate oxidase catalyses the oxidation of lactate to pyruvate and hydrogen peroxide (H_2O_2). The formed pyruvate and hydrogen peroxide under the influence of peroxidase (POD), 4-aminophenazone (4-AP) and 4-

chlorophenol form a red quinone compound. The intensity of the colour formed is proportional to the lactate concentration in the sample.



In a 96-well plate, 2 µl of supernatant were added per well. A calibration curve based in a range of lactate solutions with different concentrations was performed. After, 200 µl of lactate reagent were added to the wells and the plate was incubated for 10 minutes. The blank was made only with 200 µl of lactate reagent. Absorbance was read at 490 nm.

3.9. Cell Migration / Wound Healing Assay

The Scratch Wound Healing Assay is a simple and inexpensive technique used to study the effects of a variety of experimental conditions, like gene-knockdown or chemical compound treatment, on cell migration. In this assay, a “wound gap” is created by a scratch in a cell monolayer, followed by monitoring the “healing” of this gap by cell migration. This assay helped us to understand the migratory capacity of the cells after MCTs knockdown and it was performed as previously described [154]. The cell lines 22RV1 and DU145 were seeded in a six-well culture plate (Nunc, Thermo Scientific), at a density of 600.000 and 500.000 cells/well. After reaching 95 % of confluence, the cell monolayer was “wounded” by scraping it with a 200 µl pipette tip and washed with PBS 1x. Then, cells were covered with RPMI medium (without FBS). Specific scratching sites of the wound areas were analyzed and photographed at 0 and 24 hours. The relative migration distances were analyzed using the software QWound (developed at the ICVS by the biomedical engineering team) and data were expressed in % of control migration.

3.10. Chicken Chorioallantoic Membrane (CAM) Assay

The chicken chorioallantoic membrane (CAM) assay is a quick, technically simple, and inexpensive *in vivo* assay. After tumor development, the blood vessel network can be easily accessed, manipulated and observed providing an optimal setting to study the effect of lack of proteins, in this case MCTs, in the tumor growth as well as the formation of new blood vessels (angiogenesis). The main problem of this technique is consistency of the assays,

which implies the use of a large number of eggs. The CAM assay was performed as previously described [154,172]. Fertilized chicken eggs (Pinto Bar) were incubated at 37 ºC. On day 3 of development, after puncturing the air chamber, a hole in a specific region of the eggshell was performed and eggs were sealed with tape and returned to the incubator. On the 10th day of development, a plastic ring was placed on the CAM and the different conditions of tumor cells in study (22RV1 scramble and siMCT1, DU145 scramble and siMCT1+4) were injected inside the ring. The eggs were tapped and returned to the incubator. The effect MCT silencing on tumor growth and vascularization was assessed after 14 days of development. In the 14th day, digital images of the egg were taken in a stereomicroscope (Olympus S2 × 16), using a digital camera (Olympus DP71) and, after that, the chicken embryos were sacrificed by 10 min incubation at -80 ºC and the CAMs were dissected in order to take *ex ovo* digital images.

3.11. Statistical Analysis

All graphs and statistical analysis were performed with the Graph Pad Prism 5 software. The results were expressed as mean ± SEM. Statistical significance between two groups was assessed by t-test. P values ≤0.05 were considered statistically significant.

CHAPTER 4: RESULTS

4.1. Characterization of expression of MCTs, CD147, CD44 and metabolic markers in prostate cancer cell lines

Assessment of the role of MCTs in prostate cancer was done using two different prostate cell lines, with distinct phenotypes. 22RV1 cell line derives from a localized prostate cancer, representing therefore a less aggressive cancer, and DU145 cell line, in turn, derives from a cerebral metastasis of a prostate tumor, representing a tumor with more aggressive phenotype.

Prostate cancer cell lines were firstly assessed for the expression of different metabolic markers, namely HKII, LDH-5, GLUT-1, CAIX, PDH and PDK, as well as for MCT1, MCT2, MCT4 and their chaperones CD147 and CD44. This procedure allowed us to characterize the expression of relevant proteins on glycolytic metabolism, in order to predict if these protein will may play a role on the energetic metabolism of prostate tumor cells. This characterization was done by Western blotting for the assessing of expression levels, while the cellular localization was assessed by immunocytochemistry.

Despite Western blotting not showing the cellular localization of proteins, it is a sensitive method regarding to protein expression level. As can be seen by Figure 21, MCT1 is expressed in both cell lines, with less expression in 22RV1 cells in comparison with DU145. The expression of MCT2 was only observed in less aggressive cell line, being absent in DU145 cells. Importantly, 22RV1 cells do not express MCT4 at all, being this protein largely expressed in DU145 cell line. CD147 was present in both cell lines, although with slightly smaller expression in 22RV1, while CD44 was expressed exclusively in DU145.

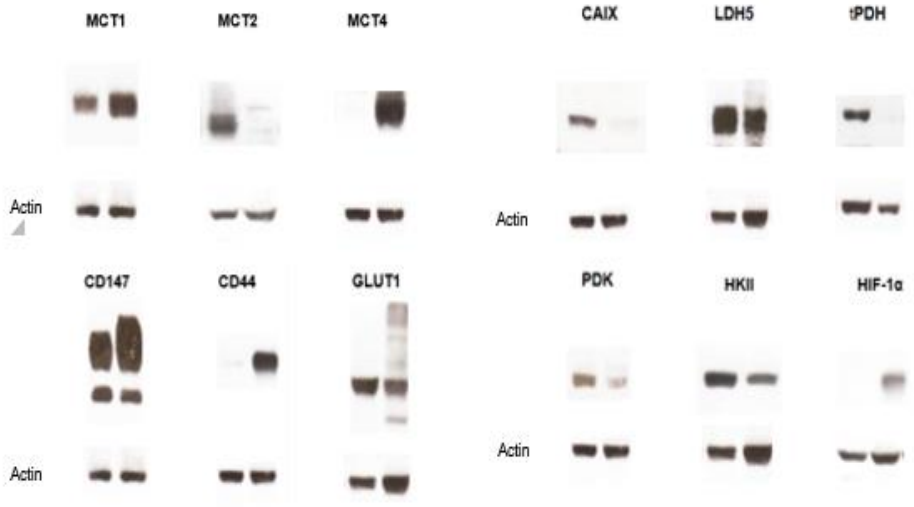


Figure 21. Western blotting analysis of the expression of MCTs and glycolytic markers in 22RV1 and DU145 cell lines. Loading order: 22RV1 / DU145.

In what regards to the selected panel of glycolytic markers, GLUT-1, LDH-5 and PDK were expressed in both 22RV1 and DU145 cells, with similar levels, however, expression of CAIX and PDH was only present in 22RV1 cells, while HKII appears both in 22RV1 and in DU145 cells, with a decrease in the second cell line. HIF-1α only showed expression on DU145 cells.

Concerning to cellular localization of the expressed proteins, as shown in the Figure 22, MCT1 was expressed in both membrane and cytoplasm of 22RV1 cells, while in DU145 no membrane expression was detected, having only a slight presence in cytoplasm of these cells. MCT2 was expressed in 22RV1 cells' plasma membrane, but not detected in DU145 cells as seen previously by Western blot results. MCT4 seems to be present in both plasma membrane and cytoplasm of DU145 cells, with a slight expression in the cytoplasm of 22RV1 cells. In what regards to the known chaperones of MCT1 and MCT4, both CD147 and CD44 were expressed in DU145 cell, exclusively at the plasma membrane. Regarding the relationship of MCTs and CD147 or CD44, it can only be seen an association of chaperones with MCT4 for DU145 cells, but not in 22RV1 which, in fact, there was no membrane expression of MCT4, in contrast with DU145 cells. However, despite no expression of either CD147 or CD44 in 22RV1 cells, some membrane expression for MCT1 could be detected.

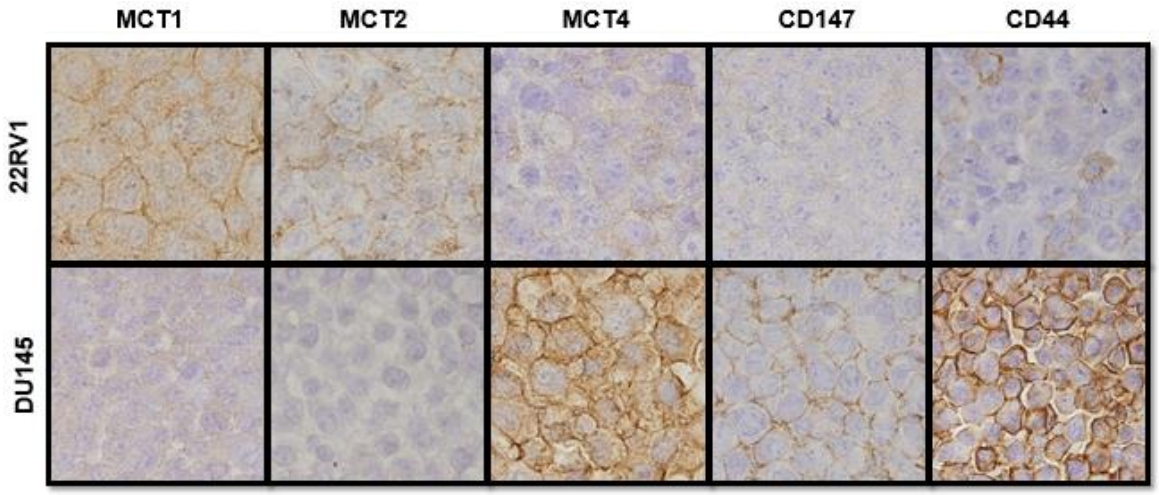


Figure 22. Expression of MCT1, MCT2 and MCT4 in 22RV1 and DU145 cell lines assessed by immunocytochemistry. The expression of CD147, chaperone of MCT1 and MCT4 was also assessed. (400x magnification)

Additionally, the expression of a panel of relevant glycolytic markers was assessed in order to evaluate the involvement of the glycolytic metabolism in the prostate cell lines under study. As can be seen in Figure 23, both cell lines expressed GLUT-1, although membrane expression for this marker only appeared in DU145 cells. CAIX, LDH-5, PDH and PDK were

expressed in both cell lines, being HKII only expressed in 22RV1 cells and nuclear HIF-1α in DU145 cells. Importantly, LDH-5 and HKII expression was higher in 22RV1 than in DU145 cells, with a cytoplasmic staining pattern.

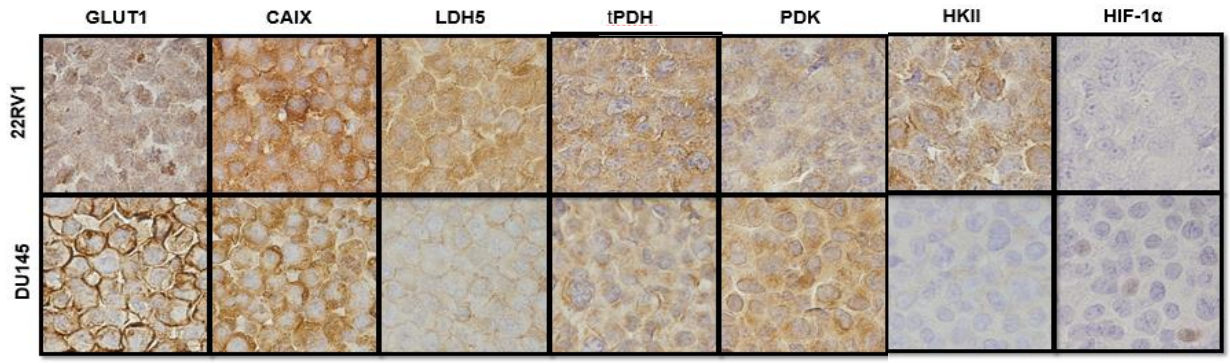


Figure 23. Expression of glycolytic markers in 22RV1 and DU145 cell lines, assessed by immunocytochemistry. (400x magnification)

4.2. Effect of MCT downregulation on cellular metabolism of prostate cells

After assessment of the expression of MCTs in 22RV1 and DU145 cells, we performed the silencing of the expressed MCTs in each cell line by siRNA. Thus, according to the expression results, for 22RV1 cell line we silenced MCT1, whereas for DU145 silenced both MCT1 and MCT4, and not each isoform separately, since it has been already observed that isoforms can perform a compensatory activity between each other.

As can be seen in the graphs of Figure 24, after silencing of MCT1, 22RV1 cells showed a significant increase in glucose consumption when compared to control, after 12 hours from the start of the test. On the other hand, DU145 cells decreased its glucose consumption at the same time point and, more significantly, after 24 hours from the beginning of the test.

In what regards to the effect of MCTs downregulation on lactate efflux by prostate cancer cells, it can be seen a very significant decrease in both cell lines, with much greater impact on 22RV1 cell line, with significant differences for both 12 and 24 hours.

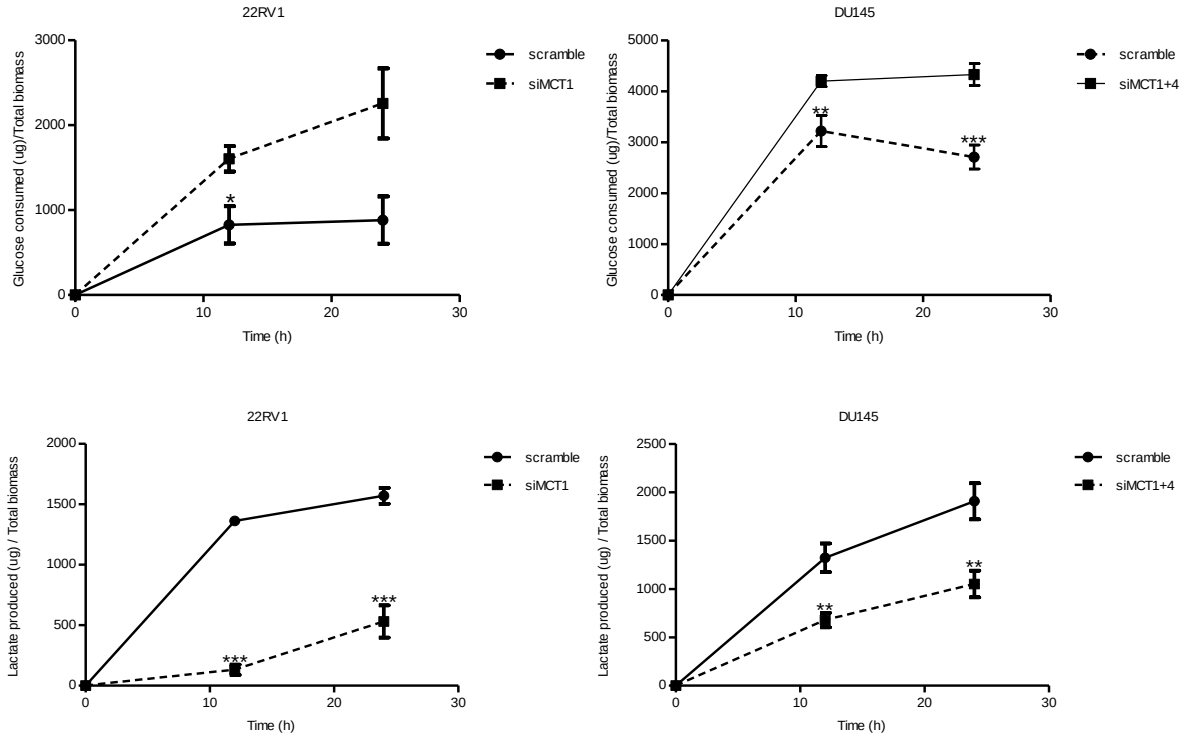


Figure 24. Effect of MCT silencing on the cellular metabolism behavior of prostate tumor cells, by assessment of glucose consumption and lactate production at 12 and 24 hours. The results represent the mean ± SEM of at least three independent experiments, each one in triplicate. * $p \leq 0.05$, ** $p \leq 0.005$, *** $p \leq 0.0001$ compared to control (scramble).

4.3. Effect of MCTs downregulation on prostate cancer cells survival

As said before, MCTs has been shown an important role in the resistance to the acidic microenvironment of tumors, thus, becomes relevant to investigate how their absence affects cancer cell survival.

In order to study the role of MCTs on tumour cells survival, viability tests were performed, using the Sulforhodamine B assay.

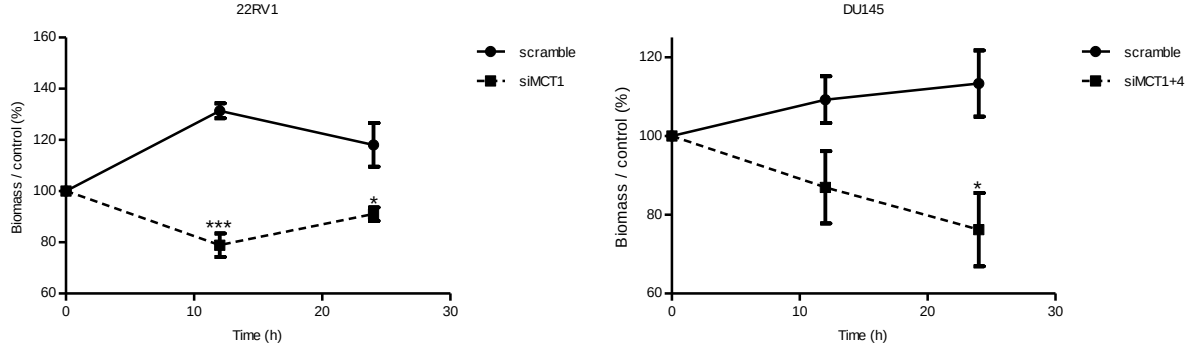


Figure 25. Effect of MCT silencing on the percentage of live cell biomass after 12 and 24 hours. Results represent the mean ± SEM of two independent experiments, each one in triplicates. * $p \leq 0.05$, *** $p \leq 0.0001$ compared to control (scramble).

The response of downregulation in terms of viable prostate cancer cells was measured after 12 and 24 hours, for both cell lines. In the graphs of figure 25, can be seen that, comparing to controls from both cell lines, MCT1 downregulation in 22RV1 cells leads to a significant decrease in viable cellular biomass until 12 hours, being this effect slightly reduced from 12 hours onwards, once from here the cells with silenced MCT1 seems to recover their ability to grow. In the case of DU145 with downregulated MCT1 and MCT4, although the effect on cell viability is less significant than those detected in 22RV1 cell line at 12 hours, a continuous effect can be seen on cell viability in comparison to the control, in contrast to what happens in 22RV1 cells.

4.4. Effect of silencing of MCTs on proliferation of prostate cancer cells

Tumor cells acquire distinct capabilities in comparison with normal cells, being one of them, as referred before, the capability to proliferate indefinitely. Adding to this, monocarboxylate transporters are proven to be involved in cell proliferation, since they cause a microenvironmental acidity, by exporting a proton along with the lactate molecule which, despite giving strength to tumor cells, is harmful for the surround normal cells, allowing tumor cells to grow. Thus, the effect of silencing of MCT1/4 on the proliferation of prostate cancer cells was analyzed in order to assess the implication of MCTs in the proliferation capacity of prostate cells. This analysis was performed by the BrdU assay, by measuring BrdU incorporation during DNA synthesis after 24 hours (Figure 26).

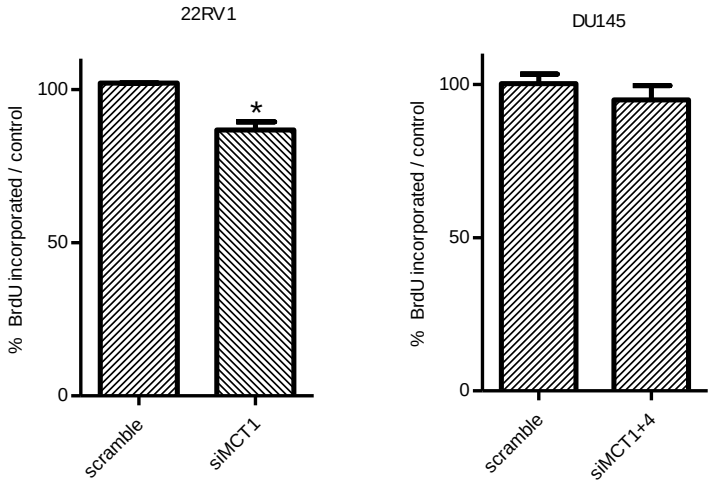


Figure 26. Effect of MCT silencing on prostate tumor cell proliferation, assessed by BrdU incorporation in DNA of prostate cell lines at 24 hours. Results represent the mean ± SEM of two independent experiments, each one in triplicate. ***** $p \leq 0.05$ compared to control (scramble).

It is possible to observe that silencing of MCT1 in 22RV1 cells promoted a significant reduction on cellular proliferation compared to the control. On the other hand, the silencing of MCT1+4 in DU145, did not produce any effect in terms of proliferation, comparing to control.

4.5. Effect of MCTs downregulation on migration capacity of prostate cancer cells

As it is known, the majority of cancer cells have the ability to migrate through the body, from the original organ to others, adjacent or non-adjacent, which contribute to metastasis, instilling difficulties in the treatment and increasing mortality rates. Thus, constituting migration an important factor when we talk about cancer, we analyzed the effect of the silencing of MCTs on the migration capacity of prostate cancer cells. For that, we used the scratch-wound healing assay for 22RV1 and DU145 cells with downregulated MCTs and assess the effect for 24 and 48 hours.

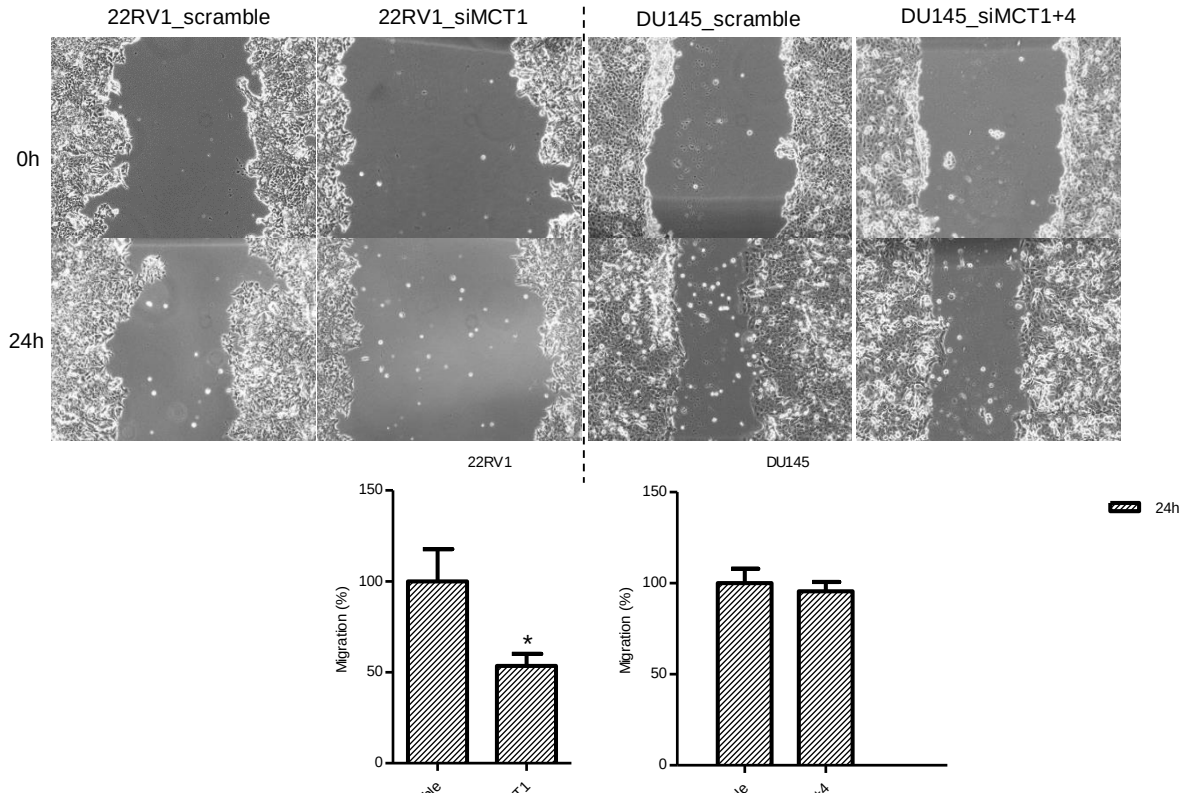


Figure 27. Inhibitory effect of MCT silencing on 22RV1 and DU145 cells migration capacity. Above, **r**epresentative pictures of the migratory capacity of 22RV1 and DU145 cells, both controls and silenced conditions for each cell line. Below, graphs representing the respective percentage of cell migration inhibition by MCT silencing for 24 hours. Results represent the mean ± SEM of at least three independent experiments. * $p \leq 0.05$ compared to control (scramble).

The results obtained from this assessment (Figure 27), showed that the migration capacity of 22RV1 cells was significantly inhibited when MCT1 was downregulated. In contrast, DU145 cells continued to migrate, in the same way, even without expression of MCT1 and MCT4.

4.6. Effect of MCTs downregulation on *in vivo* tumor formation capacity

The complexity of a living system cannot be achieved through *in vitro* studies. In cancer research, as in several other research areas, *in vitro* studies give us a view of the effects and mechanisms that are probable, however they are not able to mimic the real involvement of the human body and all the constrains underlying it. Then, and before proceeding to human clinical trials, it turns necessary to use *in vivo* models in order to confirm the previously achieved *in vitro* results. In this work, we used de chicken embryo chorioallantoic membrane (CAM) as *in vivo* model for induction of prostate tumors using cell lines, in order to assess the effect of MCT silencing in the capacity of prostate cells to give rise to tumors, as well as to have a first vision on the recruitment and formation of blood vessels into the formed tumor (i.e. angiogenesis).

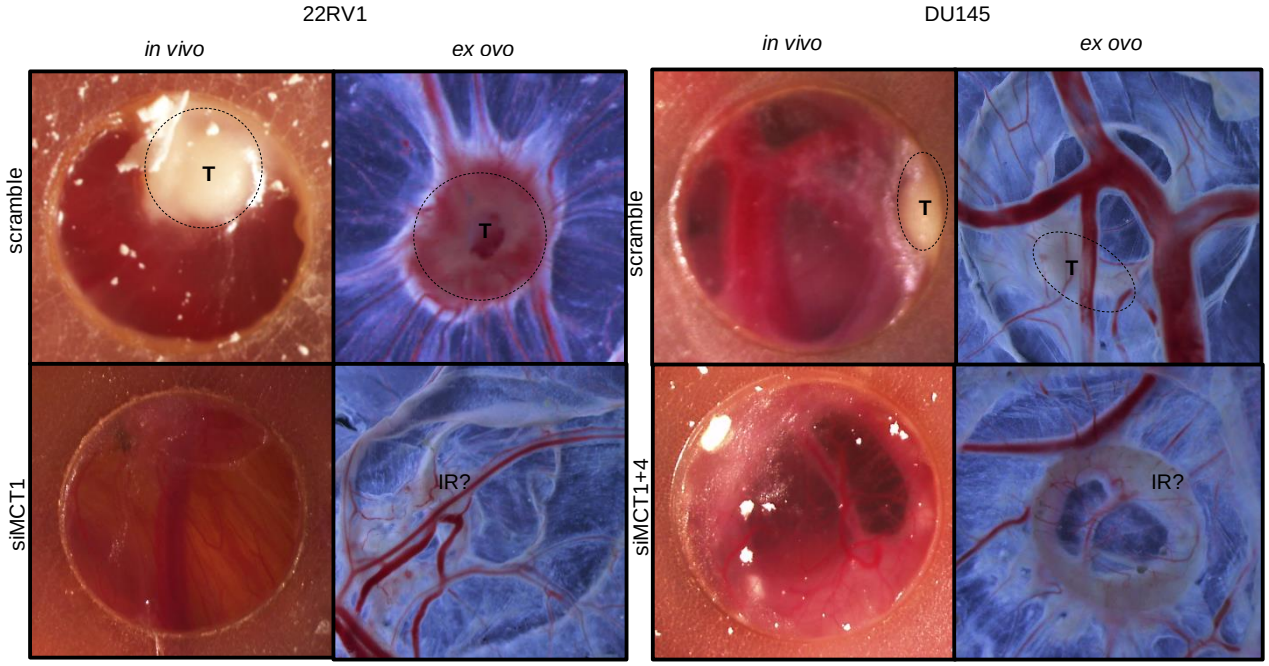


Figure 28. *In vivo* induction of prostate tumors and the effect of MCTs silencing in tumor formation after four days of injection at tenth day of incubation. E*x ovo* pictures is also shown. 10x magnification. Abbreviations: T – tumor; IR – inflammatory response (?).

Control conditions (scramble) of Figure 29, effectively show that both 22RV1 and DU145 cells have the capability of forming *in vivo* 3D tumors, despite that the tumors formed by the metastasis-derived cell line being smaller. However, only control samples were able to form visible tumors in the CAM; in fact, when we look at the conditions with silenced MCTs, the growth of tumors to a visible size was strongly inhibited for both cell lines.

Additionally, silenced conditions of both cell lines seem to provoke some kind of inflammatory reaction on the cell injection site of CAM, especially in the condition with silenced MCTs in DU145 cells, since we observed in this area, the presence of a liquid milky substance, with a distinct consistency from that observed in tumors of control samples.

In what regards to blood vessel formation or recruiting by formed tumors, it can be seen a clear blood vessel formation in the 22RV1 cells-derived tumor, but not so clearly on the tumor formed by DU145 cells.

CHAPTER 5: GENERAL DISCUSSION

General Discussion

The searching for effective treatments against cancer has constitute an increasing research area along the last years, being the “core” of this challenge, the achievement of a way to specifically kill cancer cells, while normal cells are spared. The increasing knowledge regarding the understanding of tumor biology has greatly contributed to the advancement in this area, as it has allowed the identification of several distinct characteristics between normal and tumoral cells, which therefore has paved the way for new potential targets which may be used in the treatment of this disease.

The known special capabilities of cancer cells giving rise to tumor, in a general way, the potential to grow unreservedly, to resist to mechanisms of cell dead, to evade the immune response and, importantly, to reprogram its metabolism allowing it to withstand the adversities of the resultant microenvironment, constitute a panel of distinctive features which can be used against themselves. The recognition of this last feature of cancers (i.e. reprogramming energy metabolism), has led to deeper studies that have led, in turn, to the discovery of new putative targets for cancer therapy, including the monocarboxylate transporters, important players in the maintenance of the glycolytic metabolism that, along with the phenomenon called “Warburg effect”, is a proven characteristic of most tumors [24,26].

With this study, we tried explore MCTs as targets for a targeted therapy against prostate cancer which is, currently, the second most frequently diagnosed cancer of men and the fifth most common cancer overall [1].

5.1. *Metastatic prostate cancer is more glycolytic than localized prostate cancer*

The Warburg effect, that is the adoption of a glycolytic metabolism by tumoral cells, even in normoxia conditions constitutes a widely described event in several types of cancer [31]. Associated to this, there is an increase in lactate production and, therefore, an upregulation of its main transporters, MCTs, which are already described in many tumor types [127,129,130,136,147-153,155,158]. Particularly, in prostate cancer, the information about the role of MCTs is still scarce. A study by Hao *et al.* [151] on human samples of prostate cancer described a wide expression of MCT1 and MCT4 in prostate tumors, contrasting with another recent study from Pértega-Gomes and colleagues [173], which

described an upregulation of MCT2 and MCT4, but not MCT1, when compared to normal prostate tissues, being the expression of MCT4, along with CD147, associated with tumoral progression to malignant phenotypes and with poor prognosis of patients. In the present study, we assessed MCT expression in cell lines, representing less (22RV1) and more aggressive (DU145) phenotypes of prostate cancer. The results showed that the metastasis-derived cell line, DU145, had a higher expression of MCT4, CD147 and CD44 when compared to the localized prostate tumor-derived cell line, 22RV1, which has, in turn, higher expressions of MCT1 and MCT2. The higher expression of MCT4 and chaperones in DU145 cells was in fact concordant with the described by Pértega-Gomes, about the correlation of MCT4 and CD147 with more aggressive phenotypes of prostate cancer [173]. In addition, we support the role of CD147 and CD44 as chaperone for MCT4, since MCT4 was localized in plasma membrane in these cells, with co-expression of CD147 and CD44, as it would be expected from glycolytic cells. Moreover, consistent with the glycolytic phenotype proposed for DU145 cells, along with membrane expression of MCT4, we saw an evident membrane expression of GLUT-1, supporting its role in glucose uptake. Moreover, once HIF-1α regulates positively a wide panel of proteins in the context of altered metabolism of cancer cells, namely GLUT-1 HKII, PDK, PDH, LDH, CAIX and MCT4 [65], it would be expected that DU145 cell line had a higher expression of these proteins, since we saw nuclear expression of HIF-1α in this cell line. In fact, besides the expression of LDH, PDK and HKII which was similar in both cell lines, a relationship between HIF-1α and those proteins can be seen in DU145 cells, which shows membrane expression of GLUT-1 and MCT4, involved in glucose uptake and lactate efflux, respectively.

In contrast to DU145, 22RV1 cells do not show any expression of MCT4 and GLUT-1 expression was cytoplasmic. Importantly, there was a cytoplasmic expression of both MCT1 and MCT2, in 22RV1 cells, which may be related with the described role of these proteins in lactate oxidation complex to maintain the organelle redox status, meaning that their cytoplasmic expression could be associated to mitochondria and/or peroxisome [111,112,114], consistent with the idea of a more oxidative cell line. In addition, 22RV1 cells shows a higher expression of PDH in comparison with DU145 cells, which points once again to a more oxidative phenotype, since PDH is responsible for the conversion of pyruvate resulting from the glycolytic pathway, into acetyl-CoA, which is directed to mitochondria for oxidative phosphorylation [29,30]. This is in line with what was said before about the reason for the presence of cytoplasmic MCT1/2 in this cell line.

On the other hand, despite with low level, some membrane expression of MCT1 can be seen in 22RV1 cells. Taking that into account, although this line seems to be more oxidative than glycolytic, it expressed some levels of glycolytic metabolism-associated proteins, such as GLUT1, PDK, HKII and LDH so, the lack of MCT4 in these cells may be compensated by MCT1 for efflux the lactate produced, once it is an isoform with intermediated affinity for the substrate and so, it can perform both the uptake and export of lactate [67]. Additionally, since the extracellular acidity is a feature of most cancers, the presence of CAIX in 22RV1 cells can also compensate the lack of MCT4 in the role of microenvironmental acidifier [58,63]. Moreover, giving the membrane expression of MCT1 in 22RV1 cells and the lack of membrane CD147 or CD44, it can be hypothesize that some other protein, yet undescribed, can be performing the role of chaperone for MCT1.

In short, from the results of the models used, it appears that the more aggressive prostate cancer phenotype is associated with a more glycolytic metabolism, confirming the effect of the known metabolic adaptations along the carcinogenesis [31].

5.2. *MCT downregulation is harmful to localized prostate cancer in vitro*

Accordingly with the available information in the literature, MCTs are involved in a number of roles in the human body but, importantly, they are upregulated in cancer, which is generally associated with increased growth of tumors, as well as aggressiveness features, such as high proliferation rates and migration capability between different parts of the body, among others [37,38]. In prostate cancer, studies has been performed showing the upregulation of MCTs in human samples of prostate cancer [151,158] and, in fact, in this study we confirmed the high expression of MCT4 in metastatic (i.e. more aggressive) cells originated from prostate, associating metastatic tumor with glycolytic metabolism, while for non-metastatic cells (i.e. less aggressive, localized tumor) we saw an association with MCT1 and MCT2 expression, being these cells less glycolytic.

Following the rationale that MCTs may be important for survival and aggressive behavior of cancer cells, and once they are highly expressed in those cells, we performed a series of *in vitro* assays in order to investigate the contribution of these MCTs to prostate cancer survival and features of aggressiveness, performing their silencing by siRNA. It would be expected that, facing the lack of MCTs, lactate would be accumulated in cell inner space, with prejudice for cell survival and integrity, as well as for the energetic metabolism

adopted by these cells (i.e. glycolytic) which, theoretically, would be blocked by a kind of negative feedback.

In a general way, silencing of MCTs had a greater impact on 22RV1 than DU145 cells. MCT knockdown led, in both cell lines, to a decrease in lactate production as would be expected, since MCTs are the responsible for that task [91]. In DU145 cells, as expected, this blocking in lactate efflux consequently led to a significant reduction in glucose consumption by these cells, confirming the idea of negative feedback regulation. However, and contrasting with the expected, MCTs knockdown led to the increase in glucose consumption by 22RV1 cells. There may be some explanations for this increase when we silence MCTs, being all of them related with the activation, or upregulation, of some other energetic pathway, in face of the blocking of glycolysis, for which glucose has been redirected. One hypothesis is that 22RV1 cells, which we showed already to be more oxidative than glycolytic, in face of the inability to export lactate, may increase even more the oxidative phosphorylation pathway, converting pyruvate to acetyl-CoA instead converting it to lactate. On the other hand, another possible explanation is that glucose continuing to be consumed and is deviated for the pentose-phosphate pathway, which originate, among others, NADPH, that have a role in the prevention of oxidative stress, as well as in the *de novo* lipogenesis, which fatty acids produced can be used subsequently to produce energy through fatty acid β-oxidation, an already described prostate cancer-associated pathway [174,175]. Recently, *de* Bari and colleagues firstly described the occurrence of a D-lactate mitochondrial metabolism in prostate cancer, as a way of energy production. This D-lactate can enter in the cell via monocarboxylate transporters, like L-lactate, but importantly, it can be produced in the cytosol through the methylglyoxal pathway, being then oxidized in the inner membrane of mitochondria by D-LDH (D-lactate dehydrogenase). Moreover, in the same study, mitochondrial oxidation of D-Lactate proved to contribute to both ROS scavenging and fatty acid synthesis, becoming important in the maintenance of cell viability and proliferation [176]. Having this in mind, it could be reasonable to hypothesize that, with lack of MCTs and functional glycolytic pathway, an activation or upregulation of methylglyoxal pathway could be performed. This will allow the maintenance of cell viability and redox status, at the same time that the production of fatty acids is induced to be used by β-oxidation for energy production.

5.3. *MCT expression is required for the in vivo prostate tumors growth*

The formation of a tumor in a living complex system, requires much more skills by tumor cells than those in a monolayer culture, as in case of the *in vitro* studies. A living system includes an entire environmental involvement, with different types of cells, an associated immune system, limitation of nutrients and oxygen that cannot be externally controlled, angiogenic capabilities and, importantly, a 3D configuration that can make the difference comparing with a 2D *in vitro* culture. Thus, *in vivo* studies are essential for the real perception of the effects of experiments carried out in research, including in cancer.

In this work, we performed a series of *in vitro* studies using prostate cancer cell lines, and we demonstrate that the inhibition of the expression of MCTs in these cells has an effect on several malignant features of prostate cells, namely on survival, proliferation, migration and the adopted energetic metabolism. Therefore, taking into account these results, we wanted to make a first approach to study the effectiveness of MCT silencing in the formation and growth of prostate tumors in a living system, and for that we performed the tumor induction in the CAM.

A very deep analysis was not necessary to verify that tumor growth was severely compromised when the expression of MCTs was inhibited. In fact, in the samples where MCTs have been silenced, the tumors did not appear to have formed at all, however, we have preliminary results (data not shown) where for the samples, the formation of micro-tumors was checked and they were only visible under microscopic analysis after processing and haematoxylin-eosin staining. This indicates that the lack of MCTs have the ability to inhibit tumor growth but not the formation of tumors.

Importantly, as previously said, it is described that microenvironmental lactate is associated with VEGF production, the major angiogenic factor [59] and, in fact, we observed a great blood vessels formation in tumors of untreated cells, with normal MCT levels, and so with normal glycolytic rates and lactate export. In addition, there was a more evident angiogenesis capacity in 22RV1 cells, which can be due to the characteristic of primary tumor of these cells. Since these are cells from a primary tumor, they will have all the capabilities to settle and grow in a place, hence the greatest growth in the CAM, comparing to the metastatic cells. Thus, 22RV1 cells, through the recruitment of blood vessels can capture the nutrients they need to survive, which allow a greater growth. On the other hand, with metastatic DU145 cells, although more glycolytic and so greater producer of lactate which would have an influence on angiogenesis they may have lost some of their capacity

for attach [177-180] so they can travel through the body, and may therefore lose advantage in local growth and subsistence.

Interestingly, in the MCT-silenced samples appear what seems to be an inflammatory response, detected by the presence of a substance with a liquid milky appearance, spread at the site of cell injection on CAM. This suggestion could gain some strength if we think of what was said earlier about T-cell inactivation by lactate. In fact, T-cell activation is dependent on high rates of glycolysis and therefore on the rapid efflux of lactate [86], being its metabolism and function compromised in the presence of high extracellular concentrations of lactate which will exert a negative feedback and block lactate efflux, decreasing the immune response against tumor [70]. Having this in mind, the MCT silencing, with its consequent decrease in lactate efflux from tumor cells, may have provided an opportunity for the intervention of the immune system against tumor cells, contributing also to the lack in formation of a substantial tumor in the silenced conditions for MCTs.

In summary, with these study we demonstrate that MCTs are expressed in prostate tumor cells, in an aggressiveness-dependent way for each isoform. Additionally, using *in vitro* prostate cancer models, we demonstrated the effectiveness of MCT1 and MCT4 expression inhibition in counteracting various characteristics of aggressiveness of prostate tumor cells, as well as undermine the viability of these cells. Moreover, we showed the great potential of the inhibition of MCT expression in reducing tumor growth and angiogenesis *in vivo*. Thus, despite further studies still be needed, we showed here that MCTs can be powerful targets to consider in the development of new targeted therapies in the treatment of prostate cancer.

FUTURE PERSPECTIVES

In this work we resorted to cell lines to assess the role of MCTs in prostate cancer, along with some metabolic markers known for being associated with glycolysis, and we suggested that MCTs have different roles in prostate cancer, depending on the isoforms expressed in each type of cells. Moreover, we assessed the effect of the inhibition of MCT expression in these cell lines and demonstrated a decrease on cell survival and energetic metabolism, as well as on proliferation and migration capacity of less aggressive phenotypes of prostate cancer. Importantly, MCT silencing showed *in vivo* capability of decrease tumor growth and angiogenesis. However, although the potential of MCTs as therapeutic targets in prostate cancer has been demonstrated, further studies will be needed to achieve the real value and the effectiveness of MCTs in this therapeutic approach and to take a step forward in its use in humans.

Firstly, it will be necessary to augment the panel of prostate cell lines in order to confirm the results obtained in this work for MCTs and metabolic markers expression as well as for the effects of MCT silencing. Importantly, “normal” cell lines of prostate need to be included in this work to assess the expression and effects of MCT silencing in normal prostate cells, in order to evaluate the specificity of these therapeutic approach for cancer cells, sparing normal prostate cells.

Additionally, once we saw a decrease in cell viability due to MCT silencing, by measurement of viable cells biomass in culture, it becomes relevant to perform studies on cell death, exploring both apoptosis induction and presence of necrosis, in order to confirm the effectiveness of MCT silencing in kill cancer cells and not only in decreasing its proliferation.

Moreover, it could be important to use activity inhibitors of MCTs in order to perform the same assessment as which was performed for expression inhibition. It is needed to assess the effects of such inhibition, in comparison to the expression inhibition already performed, once the approaching of an MCT-targeted therapy in humans could be easier using activity inhibitors.

Additionally, along with MCT inhibition, is important to achieve the inhibition of molecular chaperones of MCTs, like CD147 and CD44, trying to increase the potency of the treatment. Moreover, new regulators of MCTs need to be explored, both molecular (once our expression results suggest the existence of other chaperones, besides CD147 or CD44) and epigenetic, which stay poorly explored.

No less important, since hypoxia is a widely observed condition in tumors and it is known its regulation on MCTs and other glycolytic proteins, it could be interesting to perform our studies under hypoxia conditions.

Importantly, we proposed that, due to the inhibition of MCTs and consequent blocking on lactate efflux, cancer cells may activate or enhance other pathways to produce the energy they need. Thus, to check this possibility and identify these putative pathways, it could be necessary to evaluate the expression of specific proteins of the proposed pathways in prostate cell lines, comparing whether there is overexpression in the tumor cells compared to normal cells.

Finally, after all these assessments, a more complex *in vivo* model need to be used, such as a mouse model, to ascertain all effects in an organism closest to our true purpose of treatment: the human prostate cancer.

REFERENCES

References

1. Globocan. 2002 Database. http://www-dep.iarc.fr/ . 2009.
2. U.S.Department of Health and Human Services NCI. Understanding prostate changes: a health guide for men. 2011.
3. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 2010; 127: 2893-917.
4. Edge SB, Compton CC. The American Joint Committee on Cancer: the 7th edition of the AJCC cancer staging manual and the future of TNM. Ann Surg Oncol 2010; 17: 1471-4.
5. Jewett HJ. The present status of radical prostatectomy for stages A and B prostatic cancer. Urol Clin North Am 1975; 2: 105-24.
6. Ohori M, Wheeler TM, Scardino PT. The New American Joint Committee on Cancer and International Union Against Cancer TNM classification of prostate cancer. Clinicopathologic correlations. Cancer 1994; 74: 104-14.
7. Epstein JI, Allsbrook WC, Jr., Amin MB, Egevad LL. The 2005 International Society of Urological Pathology (ISUP) Consensus Conference on Gleason Grading of Prostatic Carcinoma. Am J Surg Pathol 2005; 29: 1228-42.
8. Heidenreich A, Aus G, Bolla M, Joniau S, Matveev VB, Schmid HP, Zattoni F. EAU guidelines on prostate cancer. Eur Urol 2008; 53: 68-80.
9. Holmberg L, Bill-Axelson A, Steineck G, Garmo H, Palmgren J, Johansson E, Adami HO, Johansson JE. Results from the Scandinavian Prostate Cancer Group Trial Number 4: a randomized controlled trial of radical prostatectomy versus watchful waiting. J Natl Cancer Inst Monogr 2012; 2012: 230-3.
10. Kumar S, Shelley M, Harrison C, Coles B, Wilt TJ, Mason MD. Neo-adjuvant and adjuvant hormone therapy for localised and locally advanced prostate cancer. Cochrane Database Syst Rev 2006; CD006019.
11. Bolla M, Collette L, Blank L, Warde P, Dubois JB, Mirimanoff RO, Storme G, Bernier J, Kuten A, Sternberg C, Mattelaer J, Lopez TJ, Pfeffer JR, Lino CC, Zurlo A, Pierart M. Long-term results with immediate androgen suppression and external irradiation in patients with locally advanced prostate cancer (an EORTC study): a phase III randomised trial. Lancet 2002; 360: 103-6.

12. Bolla M, Gonzalez D, Warde P, Dubois JB, Mirimanoff RO, Storme G, Bernier J, Kuten A, Sternberg C, Gil T, Collette L, Pierart M. Improved survival in patients with locally advanced prostate cancer treated with radiotherapy and goserelin. N Engl J Med 1997; 337: 295-300.

13. Ash D, Flynn A, Battermann J, de RT, Lavagnini P, Blank L. ESTRO/EAU/EORTC recommendations on permanent seed implantation for localized prostate cancer. Radiother Oncol 2000; 57: 315-21.

14. Machtens S, Baumann R, Hagemann J, Warszawski A, Meyer A, Karstens JH, Jonas U. Long-term results of interstitial brachytherapy (LDR-Brachytherapy) in the treatment of patients with prostate cancer. World J Urol 2006; 24: 289-95.

15. Bottke D, Wiegel T. Adjuvant radiotherapy after radical prostatectomy: indications, results and side effects. Urol Int 2007; 78: 193-7.

16. Aus G. Current status of HIFU and cryotherapy in prostate cancer--a review. Eur Urol 2006; 50: 927-34.

17. Iversen P, Tyrrell CJ, Kaisary AV, Anderson JB, Baert L, Tammela T, Chamberlain M, Carroll K, Gotting-Smith K, Blackledge GR. Casodex (bicalutamide) 150-mg monotherapy compared with castration in patients with previously untreated nonmetastatic prostate cancer: results from two multicenter randomized trials at a median follow-up of 4 years. Urology 1998; 51: 389-96.

18. Loblaw DA, Virgo KS, Nam R, Somerfield MR, Ben-Josef E, Mendelson DS, Middleton R, Sharp SA, Smith TJ, Talcott J, Taplin M, Vogelzang NJ, Wade JL, III, Bennett CL, Scher HI. Initial hormonal management of androgen-sensitive metastatic, recurrent, or progressive prostate cancer: 2006 update of an American Society of Clinical Oncology practice guideline. J Clin Oncol 2007; 25: 1596-605.

19. Hussain M, Tangen CM, Higano C, Schelhammer PF, Faulkner J, Crawford ED, Wilding G, Akdas A, Small EJ, Donnelly B, MacVicar G, Raghavan D. Absolute prostate-specific antigen value after androgen deprivation is a strong independent predictor of survival in new metastatic prostate cancer: data from Southwest Oncology Group Trial 9346 (INT-0162). J Clin Oncol 2006; 24: 3984-90.

20. Yagoda A, Petrylak D. Cytotoxic chemotherapy for advanced hormone-resistant prostate cancer. Cancer 1993; 71: 1098-109.

21. Gibbons RP. Prostate cancer. Chemotherapy. Cancer 1987; 60: 586-8.

22. Petrylak DP, Tangen CM, Hussain MH, Lara PN, Jr., Jones JA, Taplin ME, Burch PA, Berry D, Moinpour C, Kohli M, Benson MC, Small EJ, Raghavan D, Crawford ED.

Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. N Engl J Med 2004; 351: 1513-20.

23. Sartor AO. Progression of metastatic castrate-resistant prostate cancer: impact of therapeutic intervention in the post-docetaxel space. J Hematol Oncol 2011; 4: 18.

24. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144: 646-74.

25. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell 2009; 136: 823-37.

26. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100: 57-70.

27. Lazebnik Y. What are the hallmarks of cancer? Nat Rev Cancer 2010; 10: 232-3.

28. Vander Heiden MG. Targeting cancer metabolism: a therapeutic window opens. Nat Rev Drug Discov 2011; 10: 671-84.

29. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 2008; 7: 11-20.

30. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009; 324: 1029-33.

31. Gatenby RA, Gillies RJ. Why do cancers have high aerobic glycolysis? Nat Rev Cancer 2004; 4: 891-9.

32. WARBURG O. On the origin of cancer cells. Science 1956; 123: 309-14.

33. Jadvar H, Alavi A, Gambhir SS. 18F-FDG uptake in lung, breast, and colon cancers: molecular biology correlates and disease characterization. J Nucl Med 2009; 50: 1820-7.

34. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer 2011; 11: 85-95.

35. Franzius C. FDG PET: advantages for staging the mediastinum? Lung Cancer 2004; 45 Suppl 2: S69-S74.

36. Phelps ME. PET: the merging of biology and imaging into molecular imaging. J Nucl Med 2000; 41: 661-81.

37. Gillies RJ, Robey I, Gatenby RA. Causes and consequences of increased glucose metabolism of cancers. J Nucl Med 2008; 49 Suppl 2: 24S-42S.

38. Kroemer G, Pouyssegur J. Tumor cell metabolism: cancer's Achilles' heel. Cancer Cell 2008; 13: 472-82.

39. DeBerardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab 2008; 7: 11-20.

40. Vander Heiden MG, Cantley LC, Thompson CB. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009; 324: 1029-33.

41. Weljie AM, Jirik FR. Hypoxia-induced metabolic shifts in cancer cells: moving beyond the Warburg effect. Int J Biochem Cell Biol 2011; 43: 981-9.

42. Koppenol WH, Bounds PL, Dang CV. Otto Warburg's contributions to current concepts of cancer metabolism. Nat Rev Cancer 2011; 11: 325-37.

43. Jose C, Bellance N, Rossignol R. Choosing between glycolysis and oxidative phosphorylation: a tumor's dilemma? Biochim Biophys Acta 2011; 1807: 552-61.

44. Nakajima EC, Van HB. Metabolic symbiosis in cancer: refocusing the Warburg lens. Mol Carcinog 2013; 52: 329-37.

45. Dang CV, Kim JW, Gao P, Yustein J. The interplay between MYC and HIF in cancer. Nat Rev Cancer 2008; 8: 51-6.

46. Vousden KH, Ryan KM. p53 and metabolism. Nat Rev Cancer 2009; 9: 691-700.

47. Dang CV, Semenza GL. Oncogenic alterations of metabolism. Trends Biochem Sci 1999; 24: 68-72.

48. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nat Rev Cancer 2011; 11: 85-95.

49. Gatenby RA, Gillies RJ. A microenvironmental model of carcinogenesis. Nat Rev Cancer 2008; 8: 56-61.

50. Kennedy KM, Dewhirst MW. Tumor metabolism of lactate: the influence and therapeutic potential for MCT and CD147 regulation. Future Oncol 2010; 6: 127-48.

51. Semenza GL. Hypoxia-inducible factor 1 and the molecular physiology of oxygen homeostasis. J Lab Clin Med 1998; 131: 207-14.

52. Semenza GL. Hypoxia-inducible factor 1: master regulator of O2 homeostasis. Curr Opin Genet Dev 1998; 8: 588-94.

53. Semenza GL. Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 1999; 15: 551-78.

54. Semenza GL. Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Crit Rev Biochem Mol Biol 2000; 35: 71-103.

55. Semenza GL. Hypoxia-inducible factor 1: control of oxygen homeostasis in health and disease. Pediatr Res 2001; 49: 614-7.

56. Greijer AE, van der Groep P, Kemming D, Shvarts A, Semenza GL, Meijer GA, van de Wiel MA, Belien JA, van Diest PJ, van der Wall E. Up-regulation of gene expression by hypoxia is mediated predominantly by hypoxia-inducible factor 1 (HIF-1). J Pathol 2005; 206: 291-304.

57. Cassavaugh J, Lounsbury KM. Hypoxia-mediated biological control. J Cell Biochem 2011; 112: 735-44.

58. Chiche J, Brahimi-Horn MC, Pouyssegur J. Tumour hypoxia induces a metabolic shift causing acidosis: a common feature in cancer. J Cell Mol Med 2010; 14: 771-94.

59. Kim JW, Gao P, Dang CV. Effects of hypoxia on tumor metabolism. Cancer Metastasis Rev 2007; 26: 291-8.

60. Denko NC. Hypoxia, HIF1 and glucose metabolism in the solid tumour. Nat Rev Cancer 2008; 8: 705-13.

61. Brahimi-Horn MC, Bellot G, Pouyssegur J. Hypoxia and energetic tumour metabolism. Curr Opin Genet Dev 2011; 21: 67-72.

62. Feron O. Pyruvate into lactate and back: from the Warburg effect to symbiotic energy fuel exchange in cancer cells. Radiother Oncol 2009; 92: 329-33.

63. Pastorekova S, Ratcliffe PJ, Pastorek J. Molecular mechanisms of carbonic anhydrase IX-mediated pH regulation under hypoxia. BJU Int 2008; 101 Suppl 4: 8-15.

64. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1alpha-dependent mechanism. J Biol Chem 2006; 281: 9030-7.

65. Sonveaux P, Vegran F, Schroeder T, Wergin MC, Verrax J, Rabbani ZN, De Saedeleer CJ, Kennedy KM, Diepart C, Jordan BF, Kelley MJ, Gallez B, Wahl ML, Feron O, Dewhirst MW. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. J Clin Invest 2008; 118: 3930-42.

66. Porporato PE, Dhup S, Dadhich RK, Copetti T, Sonveaux P. Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. Front Pharmacol 2011; 2: 49.

67. Pinheiro C, Longatto-Filho A, Azevedo-Silva J, Casal M, Schmitt FC, Baltazar F. Role of monocarboxylate transporters in human cancers: state of the art. J Bioenerg Biomembr 2012; 44: 127-39.

68. Dhup S, Dadhich RK, Porporato PE, Sonveaux P. Multiple biological activities of lactic acid in cancer: influences on tumor growth, angiogenesis and metastasis. Curr Pharm Des 2012; 18: 1319-30.

69. Hirschhaeuser F, Sattler UG, Mueller-Klieser W. Lactate: a metabolic key player in cancer. Cancer Res 2011; 71: 6921-5.

70. Fischer K, Hoffmann P, Voelkl S, Meidenbauer N, Ammer J, Edinger M, Gottfried E, Schwarz S, Rothe G, Hoves S, Renner K, Timischl B, Mackensen A, Kunz-Schughart L, Andreesen R, Krause SW, Kreutz M. Inhibitory effect of tumor cell-derived lactic acid on human T cells. Blood 2007; 109: 3812-9.

71. Vegran F, Boidot R, Michiels C, Sonveaux P, Feron O. Lactate influx through the endothelial cell monocarboxylate transporter MCT1 supports an NF-kappaB/IL-8 pathway that drives tumor angiogenesis. Cancer Res 2011; 71: 2550-60.

72. Quennet V, Yaromina A, Zips D, Rosner A, Walenta S, Baumann M, Mueller-Klieser W. Tumor lactate content predicts for response to fractionated irradiation of human squamous cell carcinomas in nude mice. Radiother Oncol 2006; 81: 130-5.

73. Walenta S, Mueller-Klieser WF. Lactate: mirror and motor of tumor malignancy. Semin Radiat Oncol 2004; 14: 267-74.

74. Walenta S, Wetterling M, Lehrke M, Schwickert G, Sundfor K, Rofstad EK, Mueller-Klieser W. High lactate levels predict likelihood of metastases, tumor recurrence, and restricted patient survival in human cervical cancers. Cancer Res 2000; 60: 916-21.

75. Brizel DM, Schroeder T, Scher RL, Walenta S, Clough RW, Dewhirst MW, Mueller-Klieser W. Elevated tumor lactate concentrations predict for an increased risk of metastases in head-and-neck cancer. Int J Radiat Oncol Biol Phys 2001; 51: 349-53.

76. Paschen W, Djuricic B, Mies G, Schmidt-Kastner R, Linn F. Lactate and pH in the brain: association and dissociation in different pathophysiological states. J Neurochem 1987; 48: 154-9.

77. Pertega-Gomes N, Vizcaino JR, Miranda-Goncalves V, Pinheiro C, Silva J, Pereira H, Monteiro P, Henrique RM, Reis RM, Lopes C, Baltazar F. Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer. BMC Cancer 2011; 11: 312.

78. Lu H, Forbes RA, Verma A. Hypoxia-inducible factor 1 activation by aerobic glycolysis implicates the Warburg effect in carcinogenesis. J Biol Chem 2002; 277: 23111-5.

79. Walenta S, Schroeder T, Mueller-Klieser W. Metabolic mapping with bioluminescence: basic and clinical relevance. Biomol Eng 2002; 18: 249-62.

80. Kumar VB, Viji RI, Kiran MS, Sudhakaran PR. Endothelial cell response to lactate: implication of PAR modification of VEGF. J Cell Physiol 2007; 211: 477-85.

81. Stern R, Shuster S, Neudecker BA, Formby B. Lactate stimulates fibroblast expression of hyaluronan and CD44: the Warburg effect revisited. Exp Cell Res 2002; 276: 24-31.

82. Rudrabhatla SR, Mahaffey CL, Mummert ME. Tumor microenvironment modulates hyaluronan expression: the lactate effect. J Invest Dermatol 2006; 126: 1378-87.

83. Baumann F, Leukel P, Doerfelt A, Beier CP, Dettmer K, Oefner PJ, Kastenberger M, Kreutz M, Nickl-Jockschat T, Bogdahn U, Bosserhoff AK, Hau P. Lactate promotes glioma migration by TGF-beta2-dependent regulation of matrix metalloproteinase-2. Neuro Oncol 2009; 11: 368-80.

84. Mazurek S, Grimm H, Oehmke M, Weisse G, Teigelkamp S, Eigenbrodt E. Tumor M2-PK and glutaminolytic enzymes in the metabolic shift of tumor cells. Anticancer Res 2000; 20: 5151-4.

85. DeBerardinis RJ, Mancuso A, Daikhin E, Nissim I, Yudkoff M, Wehrli S, Thompson CB. Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. Proc Natl Acad Sci U S A 2007; 104: 19345-50.

86. Frauwirth KA, Thompson CB. Regulation of T lymphocyte metabolism. J Immunol 2004; 172: 4661-5.

87. Juel C. Lactate-proton cotransport in skeletal muscle. Physiol Rev 1997; 77: 321-58.

88. Juel C, Halestrap AP. Lactate transport in skeletal muscle - role and regulation of the monocarboxylate transporter. J Physiol 1999; 517 (Pt 3): 633-42.

89. Pellerin L, Pellegri G, Bittar PG, Charnay Y, Bouras C, Martin JL, Stella N, Magistretti PJ. Evidence supporting the existence of an activity-dependent astrocyte-neuron lactate shuttle. Dev Neurosci 1998; 20: 291-9.

90. Semenza GL. Tumor metabolism: cancer cells give and take lactate. J Clin Invest 2008; 118: 3835-7.

91. Halestrap AP, Price NT. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. Biochem J 1999; 343 Pt 2: 281-99.

92. Poole RC, Halestrap AP. Transport of lactate and other monocarboxylates across mammalian plasma membranes. Am J Physiol 1993; 264: C761-C782.

93. Halestrap AP, Meredith D. The SLC16 gene family-from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. Pflugers Arch 2004; 447: 619-28.

94. Carpenter L, Poole RC, Halestrap AP. Cloning and sequencing of the monocarboxylate transporter from mouse Ehrlich Lettre tumour cell confirms its identity as MCT1 and demonstrates that glycosylation is not required for MCT1 function. Biochim Biophys Acta 1996; 1279: 157-63.

95. Broer S, Schneider HP, Broer A, Rahman B, Hamprecht B, Deitmer JW. Characterization of the monocarboxylate transporter 1 expressed in Xenopus laevis oocytes by changes in cytosolic pH. Biochem J 1998; 333 (Pt 1): 167-74.

96. Cuff MA, Lambert DW, Shirazi-Beechey SP. Substrate-induced regulation of the human colonic monocarboxylate transporter, MCT1. J Physiol 2002; 539: 361-71.

97. Kido Y, Tamai I, Okamoto M, Suzuki F, Tsuji A. Functional clarification of MCT1-mediated transport of monocarboxylic acids at the blood-brain barrier using in vitro cultured cells and in vivo BUI studies. Pharm Res 2000; 17: 55-62.

98. Poole RC, Cranmer SL, Halestrap AP, Levi AJ. Substrate and inhibitor specificity of monocarboxylate transport into heart cells and erythrocytes. Further evidence for the existence of two distinct carriers. Biochem J 1990; 269: 827-9.

99. Broer S, Broer A, Schneider HP, Stegen C, Halestrap AP, Deitmer JW. Characterization of the high-affinity monocarboxylate transporter MCT2 in Xenopus laevis oocytes. Biochem J 1999; 341 (Pt 3): 529-35.

100. Dimmer KS, Friedrich B, Lang F, Deitmer JW, Broer S. The low-affinity monocarboxylate transporter MCT4 is adapted to the export of lactate in highly glycolytic cells. Biochem J 2000; 350 Pt 1: 219-27.

101. Manning Fox JE, Meredith D, Halestrap AP. Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. J Physiol 2000; 529 Pt 2: 285-93.

102. Morris ME, Felmlee MA. Overview of the proton-coupled MCT (SLC16A) family of transporters: characterization, function and role in the transport of the drug of abuse gamma-hydroxybutyric acid. AAPS J 2008; 10: 311-21.

103. Halestrap AP. The monocarboxylate transporter family--Structure and functional characterization. IUBMB Life 2012; 64: 1-9.

104. Price NT, Jackson VN, Halestrap AP. Cloning and sequencing of four new mammalian monocarboxylate transporter (MCT) homologues confirms the existence of a transporter family with an ancient past. Biochem J 1998; 329 (Pt 2): 321-8.

105. Pinheiro C, Baltazar F. SLC16A1 (solute carrier family 16, member 1 (monocarboxylate transporter 1)). 2010. Atlas Genet Cytogenet Oncol Haematol 2010.

106. Enerson BE, Drewes LR. Molecular features, regulation, and function of monocarboxylate transporters: implications for drug delivery. J Pharm Sci 2003; 92: 1531-44.

107. Merezhinskaya N, Fishbein WN. Monocarboxylate transporters: past, present, and future. Histol Histopathol 2009; 24: 243-64.

108. Halestrap AP, Wilson MC. The monocarboxylate transporter family--role and regulation. IUBMB Life 2012; 64: 109-19.

109. Garcia CK, Brown MS, Pathak RK, Goldstein JL. cDNA cloning of MCT2, a second monocarboxylate transporter expressed in different cells than MCT1. J Biol Chem 1995; 270: 1843-9.

110. Cuff MA, Shirazi-Beechey SP. The human monocarboxylate transporter, MCT1: genomic organization and promoter analysis. Biochem Biophys Res Commun 2002; 292: 1048-56.

111. Butz CE, McClelland GB, Brooks GA. MCT1 confirmed in rat striated muscle mitochondria. J Appl Physiol 2004; 97: 1059-66.

112. McClelland GB, Khanna S, Gonzalez GF, Butz CE, Brooks GA. Peroxisomal membrane monocarboxylate transporters: evidence for a redox shuttle system? Biochem Biophys Res Commun 2003; 304: 130-5.

113. Lin RY, Vera JC, Chaganti RS, Golde DW. Human monocarboxylate transporter 2 (MCT2) is a high affinity pyruvate transporter. J Biol Chem 1998; 273: 28959-65.

114. Benton CR, Campbell SE, Tonouchi M, Hatta H, Bonen A. Monocarboxylate transporters in subsarcolemmal and intermyofibrillar mitochondria. Biochem Biophys Res Commun 2004; 323: 249-53.

115. Gallagher-Colombo S, Maminishkis A, Tate S, Grunwald GB, Philp NJ. Modulation of MCT3 expression during wound healing of the retinal pigment epithelium. Invest Ophthalmol Vis Sci 2010; 51: 5343-50.

116. Pierre K, Pellerin L. Monocarboxylate transporters in the central nervous system: distribution, regulation and function. J Neurochem 2005; 94: 1-14.

117. Murakami Y, Kohyama N, Kobayashi Y, Ohbayashi M, Ohtani H, Sawada Y, Yamamoto T. Functional characterization of human monocarboxylate transporter 6 (SLC16A5). Drug Metab Dispos 2005; 33: 1845-51.

118. Friesema EC, Jansen J, Jachtenberg JW, Visser WE, Kester MH, Visser TJ. Effective cellular uptake and efflux of thyroid hormone by human monocarboxylate transporter 10. Mol Endocrinol 2008; 22: 1357-69.

119. Kloeckener-Gruissem B, Vandekerckhove K, Nurnberg G, Neidhardt J, Zeitz C, Nurnberg P, Schipper I, Berger W. Mutation of solute carrier SLC16A12 associates with a syndrome combining juvenile cataract with microcornea and renal glucosuria. Am J Hum Genet 2008; 82: 772-9.

120. Chung W, Kwabi-Addo B, Ittmann M, Jelinek J, Shen L, Yu Y, Issa JP. Identification of novel tumor markers in prostate, colon and breast cancer by unbiased methylation profiling. PLoS One 2008; 3: e2079.

121. Kolz M, Johnson T, Sanna S, Teumer A, Vitart V, Perola M, Mangino M, Albrecht E, Wallace C, Farrall M, Johansson A, Nyholt DR, Aulchenko Y, Beckmann JS, Bergmann S, Bochud M, Brown M, Campbell H, Connell J, Dominiczak A, Homuth G, Lamina C, McCarthy MI, Meitinger T, Mooser V, Munroe P, Nauck M, Peden J, Prokisch H, Salo P, Salomaa V, Samani NJ, Schlessinger D, Uda M, Volker U, Waeber G, Waterworth D, Wang-Sattler R, Wright AF, Adamski J, Whitfield JB, Gyllensten U, Wilson JF, Rudan I, Pramstaller P, Watkins H, Doering A, Wichmann HE, Spector TD, Peltonen L, Volzke H, Nagaraja R, Vollenweider P, Caulfield M, Illig T, Gieger C. Meta-analysis of 28,141 individuals identifies common variants within five new loci that influence uric acid concentrations. PLoS Genet 2009; 5: e1000504.

122. Reimers N, Zafrakas K, Assmann V, Egen C, Riethdorf L, Riethdorf S, Berger J, Ebel S, Janicke F, Sauter G, Pantel K. Expression of extracellular matrix metalloproteases inducer on micrometastatic and primary mammary carcinoma cells. Clin Cancer Res 2004; 10: 3422-8.

123. Kirk P, Wilson MC, Heddle C, Brown MH, Barclay AN, Halestrap AP. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. EMBO J 2000; 19: 3896-904.

124. Gallagher SM, Castorino JJ, Wang D, Philp NJ. Monocarboxylate transporter 4 regulates maturation and trafficking of CD147 to the plasma membrane in the metastatic breast cancer cell line MDA-MB-231. Cancer Res 2007; 67: 4182-9.

125. Deora AA, Philp N, Hu J, Bok D, Rodriguez-Boulan E. Mechanisms regulating tissue-specific polarity of monocarboxylate transporters and their chaperone CD147 in kidney and retinal epithelia. Proc Natl Acad Sci U S A 2005; 102: 16245-50.

126. Philp NJ, Ochrietor JD, Rudoy C, Muramatsu T, Linser PJ. Loss of MCT1, MCT3, and MCT4 expression in the retinal pigment epithelium and neural retina of the 5A11/basigin-null mouse. Invest Ophthalmol Vis Sci 2003; 44: 1305-11.

127. Pinheiro C, Longatto A, Pereira SMM, Etlinger D, Moreira MAR, Jube LF, Queiroz GS, Schmitt F, Baltazar F. Monocarboxylate transporters 1 and 4 are associated with CD147 in cervical carcinoma. Disease Markers 2009; 26: 97-103.

128. Pinheiro C, Longatto-Filho A, Simoes K, Jacob CE, Bresciani CJ, Zilberstein B, Cecconello I, Alves VA, Schmitt F, Baltazar F. The prognostic value of CD147/EMMPRIN is associated with monocarboxylate transporter 1 co-expression in gastric cancer. Eur J Cancer 2009; 45: 2418-24.

129. Pinheiro C, Reis RM, Ricardo S, Longatto FA, Schmitt F, Baltazar F. Expression of monocarboxylate transporters 1, 2 and 4 in human tumours and their association with CD147 and CD44. J Biomed Biotechnol . 2010.

130. Pinheiro C, Albergaria A, Paredes J, Sousa B, Dufloth R, Vieira D, Schmitt F, Baltazar F. Monocarboxylate transporter 1 is upregulated in basal-like breast carcinoma. Histopathology . 2010.

131. Wilson MC, Meredith D, Fox JE, Manoharan C, Davies AJ, Halestrap AP. Basigin (CD147) is the target for organomercurial inhibition of monocarboxylate transporter isoforms 1 and 4: the ancillary protein for the insensitive MCT2 is EMBIGIN (gp70). J Biol Chem 2005; 280: 27213-21.

132. Nabeshima K, Iwasaki H, Koga K, Hojo H, Suzumiya J, Kikuchi M. Emmprin (basigin/CD147): matrix metalloproteinase modulator and multifunctional cell recognition molecule that plays a critical role in cancer progression. Pathol Int 2006; 56: 359-67.

133. Yan L, Zucker S, Toole BP. Roles of the multifunctional glycoprotein, emmprin (basigin; CD147), in tumour progression. Thromb Haemost 2005; 93: 199-204.

134. Iacono KT, Brown AL, Greene MI, Saouaf SJ. CD147 immunoglobulin superfamily receptor function and role in pathology. Exp Mol Pathol 2007; 83: 283-95.

135. Slomiany MG, Grass GD, Robertson AD, Yang XY, Maria BL, Beeson C, Toole BP. Hyaluronan, CD44, and emmprin regulate lactate efflux and membrane localization of monocarboxylate transporters in human breast carcinoma cells. Cancer Res 2009; 69: 1293-301.

136. Schneiderhan W, Scheler M, Holzmann KH, Marx M, Gschwend JE, Buchholz M, Gress TM, Adler G, Seufferlein T, Oswald F. CD147 Silencing Inhibits Lactate Transport and Reduces Malignant Potential of pancreatic cancer cells in in-vivo and in-vitro Models. Gut 2009; 58: 1391-8.

137. Su J, Chen X, Kanekura T. A CD147-targeting siRNA inhibits the proliferation, invasiveness, and VEGF production of human malignant melanoma cells by down-regulating glycolysis. Cancer Lett 2009; 273: 140-7.

138. Baba M, Inoue M, Itoh K, Nishizawa Y. Blocking CD147 induces cell death in cancer cells through impairment of glycolytic energy metabolism. Biochem Biophys Res Commun 2008; 374: 111-6.

139. Naor D, Nedvetzki S, Golan I, Melnik L, Faitelson Y. CD44 in cancer. Crit Rev Clin Lab Sci 2002; 39: 527-79.

140. Marhaba R, Zoller M. CD44 in cancer progression: adhesion, migration and growth regulation. J Mol Histol 2004; 35: 211-31.

141. Toole BP, Slomiany MG. Hyaluronan, CD44 and Emmprin: partners in cancer cell chemoresistance. Drug Resist Updat 2008; 11: 110-21.

142. Toole BP, Slomiany MG. Hyaluronan: a constitutive regulator of chemoresistance and malignancy in cancer cells. Semin Cancer Biol 2008; 18: 244-50.

143. Poole RC, Halestrap AP. Interaction of the erythrocyte lactate transporter (monocarboxylate transporter 1) with an integral 70-kDa membrane glycoprotein of the immunoglobulin superfamily. J Biol Chem 1997; 272: 14624-8.

144. Pinheiro C, Longatto-Filho A, Scapulatempo C, Ferreira L, Martins S, Pellerin L, Rodrigues M, Alves VA, Schmitt F, Baltazar F. Increased expression of monocarboxylate transporters 1, 2, and 4 in colorectal carcinomas. Virchows Arch 2008; 452: 139-46.

145. Koukourakis MI, Giatromanolaki A, Harris AL, Sivridis E. Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. Cancer Res 2006; 66: 632-7.

146. Lambert DW, Wood IS, Ellis A, Shirazi-Beechey SP. Molecular changes in the expression of human colonic nutrient transporters during the transition from normality to malignancy. Br J Cancer 2002; 86: 1262-9.

147. Koukourakis MI, Giatromanolaki A, Bougioukas G, Sivridis E. Lung cancer: a comparative study of metabolism related protein expression in cancer cells and tumor associated stroma. Cancer Biol Ther 2007; 6: 1476-9.

148. Ladanyi M, Antonescu CR, Drobnjak M, Baren A, Lui MY, Golde DW, Cordon-Cardo C. The precrystalline cytoplasmic granules of alveolar soft part sarcoma contain monocarboxylate transporter 1 and CD147. Am J Pathol 2002; 160: 1215-21.

149. Pinheiro C, Longatto-Filho A, Ferreira L, Pereira SM, Etlinger D, Moreira MA, Jube LF, Queiroz GS, Schmitt F, Baltazar F. Increasing expression of monocarboxylate transporters 1 and 4 along progression to invasive cervical carcinoma. Int J Gynecol Pathol 2008; 27: 568-74.

150. Chen H, Wang L, Beretov J, Hao J, Xiao W, Li Y. Co-expression of CD147/EMMPRIN with monocarboxylate transporters and multiple drug resistance proteins is associated with epithelial ovarian cancer progression. Clin Exp Metastasis 2010; 27: 557-69.

151. Hao J, Chen H, Madigan MC, Cozzi PJ, Beretov J, Xiao W, Delprado WJ, Russell PJ, Li Y. Co-expression of CD147 (EMMPRIN), CD44v3-10, MDR1 and monocarboxylate transporters is associated with prostate cancer drug resistance and progression. Br J Cancer 2010; 103: 1008-18.

152. Asada K, Miyamoto K, Fukutomi T, Tsuda H, Yagi Y, Wakazono K, Oishi S, Fukui H, Sugimura T, Ushijima T. Reduced expression of GNA11 and silencing of MCT1 in human breast cancers. Oncology 2003; 64: 380-8.

153. de Oliveira AT, Pinheiro C, Longatto-Filho A, Brito MJ, Martinho O, Matos D, Carvalho AL, Vazquez VL, Silva TB, Scapulatempo C, Saad SS, Reis RM, Baltazar F. Co-expression of monocarboxylate transporter 1 (MCT1) and its chaperone (CD147) is associated with low survival in patients with gastrointestinal stromal tumors (GISTs). J Bioenerg Biomembr 2012; 44: 171-8.

154. Miranda-Goncalves V, Honavar M, Pinheiro C, Martinho O, Pires MM, Pinheiro C, Cordeiro M, Bebiano G, Costa P, Palmeirim I, Reis RM, Baltazar F. Monocarboxylate transporters (MCTs) in gliomas: expression and exploitation as therapeutic targets. Neuro Oncol 2013; 15: 172-88.

155. Wahl ML, Owen JA, Burd R, Herlands RA, Nogami SS, Rodeck U, Berd D, Leeper DB, Owen CS. Regulation of intracellular pH in human melanoma: potential therapeutic implications. Mol Cancer Ther 2002; 1: 617-28.

156. Froberg MK, Gerhart DZ, Enerson BE, Manivel C, Guzman-Paz M, Seacotte N, Drewes LR. Expression of monocarboxylate transporter MCT1 in normal and neoplastic human CNS tissues. Neuroreport 2001; 12: 761-5.

157. Izumi H, Takahashi M, Uramoto H, Nakayama Y, Oyama T, Wang KY, Sasaguri Y, Nishizawa S, Kohno K. Monocarboxylate transporters 1 and 4 are involved in the invasion activity of human lung cancer cells. Cancer Sci 2011; 102: 1007-13.

158. Pertega-Gomes N, Vizcaino JR, Miranda-Goncalves V, Pinheiro C, Silva J, Pereira H, Monteiro P, Henrique RM, Reis RM, Lopes C, Baltazar F. Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer. BMC Cancer 2011; 11: 312.

159. Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, Pienta KJ, Rubin MA, Chinnaiyan AM. Delineation of prognostic biomarkers in prostate cancer. Nature 2001; 412: 822-6.

160. Pertega-Gomes N, Vizcaino JR, Gouveia C, Jeronimo C, Henrique RM, Lopes C, Baltazar F. Monocarboxylate transporter 2 (MCT2) as putative biomarker in prostate cancer. Prostate 2013; 73: 763-9.

161. Tennant DA, Duran RV, Gottlieb E. Targeting metabolic transformation for cancer therapy. Nat Rev Cancer 2010; 10: 267-77.

162. Pouyssegur J, Dayan F, Mazure NM. Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 2006; 441: 437-43.

163. Fang J, Quinones QJ, Holman TL, Morowitz MJ, Wang Q, Zhao H, Sivo F, Maris JM, Wahl ML. The H+-linked monocarboxylate transporter (MCT1/SLC16A1): a potential therapeutic target for high-risk neuroblastoma. Mol Pharmacol 2006; 70: 2108-15.

164. Mathupala SP, Parajuli P, Sloan AE. Silencing of monocarboxylate transporters via small interfering ribonucleic acid inhibits glycolysis and induces cell death in malignant glioma: an in vitro study. Neurosurgery 2004; 55: 1410-9.

165. Colen CB, Seraji-Bozorgzad N, Marples B, Galloway MP, Sloan AE, Mathupala SP. Metabolic remodeling of malignant gliomas for enhanced sensitization during radiotherapy: an in vitro study. Neurosurgery 2006; 59: 1313-23.

166. Gallagher SM, Castorino JJ, Philp NJ. Interaction of monocarboxylate transporter 4 with {beta}1-integrin and its role in cell migration. Am J Physiol Cell Physiol 2009; 296: C414-C421.

167. Le FR, Chiche J, Marchiq I, Naiken T, Ilc K, Murray CM, Critchlow SE, Roux D, Simon MP, Pouyssegur J. CD147 subunit of lactate/H+ symporters MCT1 and hypoxia-inducible MCT4 is critical for energetics and growth of glycolytic tumors. Proc Natl Acad Sci U S A 2011; 108: 16663-8.

168. Bueno V, Binet I, Steger U, Bundick R, Ferguson D, Murray C, Donald D, Wood K. The specific monocarboxylate transporter (MCT1) inhibitor, AR-C117977, a novel immunosuppressant, prolongs allograft survival in the mouse. Transplantation 2007; 84: 1204-7.

169. Kim HS, Masko EM, Poulton SL, Kennedy KM, Pizzo SV, Dewhirst MW, Freedland SJ. Carbohydrate restriction and lactate transporter inhibition in a mouse xenograft model of human prostate cancer. BJU Int 2012; 110: 1062-9.

170. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proc Natl Acad Sci U S A 1987; 84: 7413-7.

171. Felgner JH, Kumar R, Sridhar CN, Wheeler CJ, Tsai YJ, Border R, Ramsey P, Martin M, Felgner PL. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. J Biol Chem 1994; 269: 2550-61.

172. Martinho O, Simoes K, Longatto-Filho A, Jacob CE, Zilberstein B, Bresciani C, Gama-Rodrigues J, Cecconello I, Alves V, Reis RM. Absence of RKIP expression is an independent prognostic biomarker for gastric cancer patients. Oncol Rep 2013; 29: 690-6.

173. Pertega-Gomes N, Vizcaino JR, Miranda-Goncalves V, Pinheiro C, Silva J, Pereira H, Monteiro P, Henrique RM, Reis RM, Lopes C, Baltazar F. Monocarboxylate transporter 4 (MCT4) and CD147 overexpression is associated with poor prognosis in prostate cancer. BMC Cancer 2011; 11: 312.

174. Kruger NJ, von SA. The oxidative pentose phosphate pathway: structure and organisation. Curr Opin Plant Biol 2003; 6: 236-46.

175. Zadra G, Photopoulos C, Loda M. The fat side of prostate cancer. Biochim Biophys Acta 2013; 1831: 1518-32.

176. de BL, Moro L, Passarella S. Prostate cancer cells metabolize d-lactate inside mitochondria via a D-lactate dehydrogenase which is more active and highly expressed than in normal cells. FEBS Lett 2013; 587: 467-73.

177. Hirohashi S. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am J Pathol 1998; 153: 333-9.

178. Birchmeier W, Behrens J. Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. Biochim Biophys Acta 1994; 1198: 11-26.

179. Cavallaro U, Christofori G. Cell adhesion in tumor invasion and metastasis: loss of the glue is not enough. Biochim Biophys Acta 2001; 1552: 39-45.

180. Albelda SM. Role of integrins and other cell adhesion molecules in tumor progression and metastasis. Lab Invest 1993; 68: 4-17.

ANNEXES

Annex I

WORLD
ESTIMATED CANCER INCIDENCE, ALL AGES: BOTH SEXES

Cancer	Numbers	Crude Rate	ASR (W)	Cumulative Risk
All cancers excl. non-melanoma skin cancer	12662554	186.9	180.8	18.63
Lung	1608055	23.7	22.9	2.77
Breast	1384155	41.2	38.9	4.13
Colorectum	1235108	18.2	17.2	1.96
Stomach	988602	14.6	14.0	1.67
Prostate	899102	26.3	27.9	3.39
Liver	749744	11.1	10.8	1.24
Cervix uteri	530232	15.8	15.2	1.56
Oesophagus	481645	7.1	7.0	0.85
Bladder	382660	5.6	5.3	0.60
Non-Hodgkin lymphoma	356431	5.3	5.1	0.53
Leukaemia	350434	5.2	5.0	0.47
Corpus uteri	288387	8.6	8.2	0.96
Pancreas	278684	4.1	3.9	0.44
Kidney	273518	4.0	3.9	0.46
Lip, oral cavity	263020	3.9	3.8	0.44
Brain, nervous system	237913	3.5	3.5	0.35
Ovary	224747	6.7	6.3	0.68
Thyroid	213179	3.1	3.1	0.31
Melanoma of skin	199627	2.9	2.8	0.30
Larynx	150677	2.2	2.2	0.28
Gallbladder	145203	2.1	2.0	0.22
Other pharynx	136622	2.0	2.0	0.24
Multiple myeloma	102826	1.5	1.4	0.17
Nasopharynx	84441	1.2	1.2	0.13
Hodgkin lymphoma	67919	1.0	1.0	0.08
Testis	52322	1.5	1.5	0.12

Crude and age-standardised rates per 100,000.
Cumulative risk [0-74], percent
GLOBOCAN 2008, IARC -1.8.2013

Annex II

WORLD
ESTIMATED CANCER MORTALITY, ALL AGES: BOTH SEXES

Cancer	Numbers	Crude Rate	ASR (W)	Cumulative Risk
All cancers excl. non-melanoma skin cancer	7564802	111.7	105.6	11.11
Lung	1376579	20.3	19.3	2.29
Stomach	737419	10.9	10.2	1.18
Liver	695726	10.3	9.9	1.13
Colorectum	609051	9.0	8.2	0.87
Breast	458503	13.7	12.4	1.33
Oesophagus	406533	6.0	5.8	0.70
Cervix uteri	275008	8.2	7.8	0.87
Pancreas	266669	3.9	3.7	0.41
Prostate	258133	7.6	7.4	0.64
Leukaemia	257161	3.8	3.6	0.33
Non-Hodgkin lymphoma	191599	2.8	2.7	0.27
Brain, nervous system	174880	2.6	2.5	0.27
Bladder	150282	2.2	2.0	0.20
Ovary	140163	4.2	3.8	0.43
Lip, oral cavity	127654	1.9	1.8	0.22
Kidney	116368	1.7	1.6	0.18
Gallbladder	109587	1.6	1.5	0.16
Other pharynx	95550	1.4	1.4	0.17
Larynx	81892	1.2	1.2	0.15
Corpus uteri	73854	2.2	1.9	0.23
Multiple myeloma	72453	1.1	1.0	0.11
Nasopharynx	51609	0.8	0.8	0.08
Melanoma of skin	46372	0.7	0.6	0.07
Thyroid	35383	0.5	0.5	0.06
Hodgkin lymphoma	29902	0.4	0.4	0.04
Testis	9874	0.3	0.3	0.02

Crude and age-standardised rates per 100,000.
Cumulative risk [0-74], percent
GLOBOCAN 2008, IARC -1.8.2013

Annex III

TNM Classification of prostate cancer (1997)

Primary tumour	
Tx	Primary tumour cannot be assessed
T0	No evidence of primary tumour
T1	Clinically inapparent tumour not palpable or visible by imaging
	T1a Tumour incidental; histological finding in less than 5% of resected tissue
	T1b Tumour incidental; histological finding in over 5% of resected tissue
	T1c Tumour identified by needle biopsy because of elevated PSA
T2	Tumour confined to the prostate
	T2a Tumour involved one lobe
	T2b Tumour involves both lobes
T3	Tumour extends through the prostatic capsule
	T3a Extracapsular extension
	T3b Tumour invades seminal vesicle
T4	Tumour is fixed or invades other adjacent structures
Regional Lymph Nodes	
Nx	Not assessed
N0	No regional lymph node metastasis
N1	Regional lymph node metastasis
Distant metastasis	
Mx	Not assessed
M0	No distant metastasis
M1	Distant metastasis
	M1a Non regional lymph nodes
	M1b Bone
	M1c Other sites

American Joint Committee on Cancer Staging Manual, 5th ed. Philadelphia, JP Lippincott, 1997, pp 219–222.

Annex IV

Stage	Definition or criteria for inclusion
A	No palpable tumor; incidental finding in operative specimen
	A1 Cancer involving < 3 chips
	A2 Cancer involving ≥ 3 chips
B	Palpable tumor confined to the prostate
	B1 Prostatic nodule ≤ 2 cm confined to one lobe
	B2 Prostatic nodule > 2 cm but confined to one lobe
	B3 Prostatic nodule involving both lobes
C	Extension beyond the prostatic capsule but without evident metastasis
	C1 Tumor < 6 cm in diameter
	C2 Tumor ≥ 6 cm in diameter

Based on Whitmore WF Jr. Hormone therapy in prostatic cancer. *Am J Med* 1956; 21:697–713; and Jewett HJ. The present status of radical prostatectomy for stage A and B prostate cancer. *Urol Clin North Am* 1975; 2:105–24.